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## Defining the role of IL-15 trans-presentation by distinct cell-types during the development and homeostasis of Natural Killer and invariant Natural Killer T cells

Eliseo F. Castillo

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**DEFINING THE ROLE OF IL-15 TRANS-PRESENTATION BY DISTINCT  
CELL-TYPES DURING THE DEVELOPMENT AND HOMEOSTASIS OF  
NATURAL KILLER AND INVARIANT NATURAL KILLER T CELLS**

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A

DISSERTATION

Presented to the Faculty of  
The University of Texas  
Health Science Center at Houston  
and  
The University of Texas  
M.D. Anderson Cancer Center  
Graduate School of Biomedical Sciences  
In Partial Fulfillment

Of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

By

Eliseo Fernando Castillo, B.S., M.S.

Houston, Texas

August, 2010

To Celestee, Tristan, Dylan and Sofie

Making the impossible – Possible!



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**DEFINING THE ROLE OF IL-15 TRANS-PRESENTATION BY DISTINCT  
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Publication No. \_\_\_\_\_

Eliseo F. Castillo, B.S., M.S.

Supervisory Professor: Kimberly S. Schluns, Ph.D.

The immuno-regulatory functions displayed by NK and iNKT cells have highlighted their importance as key lymphocytes involved in innate and adaptive immunity. Therefore, understanding the dynamics influencing the generation of NK and iNKT cells is extremely important. IL-15 has been shown to provide a critical signal throughout the development and homeostasis of NK and iNKT cells; however, the cellular source of IL-15 has remained unclear. In this investigation, I provide evidence that the cell-type providing IL-15 to NK and iNKT cells via trans-presentation is determined by the tissue site and the maturation status of NK and iNKT cells. For NK cells, I revealed the non-hematopoietic compartment provides IL-15 to NK cells in the early stages of development while hematopoietic cells were crucial for the generation and maintenance of mature NK cells. Regarding iNKT cells in the thymus, IL-15 trans-presentation by non-hematopoietic cells was crucial for the survival of mature iNKT cells. In the liver, both hematopoietic and non-hematopoietic compartments provided IL-15 to both immature and mature iNKT cells. This IL-15 signal helped mediate the survival and proliferation of both NK and iNKT cells as well as induce the functional maturation of mature iNKT cells via enhanced T-bet expression. In conclusion, my work illustrates an important notion that the immunological niche of NK and iNKT cells is tightly regulated and that this regulation is meticulously influenced by the tissue microenvironment.

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## ABBREVIATIONS

$\alpha$ GalCer	alpha-Galactosylceramide
BM	Bone marrow
BrdU	5-bromo-2-deoxyuridine
CFSE	Carboxyfluorescein succinimidyl ester
CLP	common lymphoid progenitors
DC	Dendritic cells
DN	Double negative (CD4-CD8-)
DP	Double positive (CD4+CD8+)
iIEL	intestinal intraepithelial lymphocytes
IFN	Interferon
IL	Interleukin
$\gamma$ C	common gamma chain subunit
GVHD	Graft versus host disease
GVT	Graft versus Tumor
HLA	human leukocyte antigens
HSCT	hematopoietic stem cell transplantation
KIR	killer-cell immunoglobulin-like receptors
LPS	Lipopolysaccharide
LV	Liver
iNKT	invariant Natural Killer T cells
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex

MCMV	Murine Cytomegalovirus
mTEC	medullary Thymic epithelial cells
NK	Natural Killer cells
NKp	Natural Killer precursors
RKO	IL-15R $\alpha^{-/-}$
SP	Spleen
TCR	T cell receptor
Tg	Transgenic
THY	Thymus
WT	C57BL/6 mice
X-SCID	X-linked severe combined immunodeficiency



## CHAPTER 1

### REVIEW OF RELAVANT LITERATURE

#### 1.1 Cytokine Biology.

Multiple modes of cellular communication exist between various cells of the immune system and a class of proteins referred to as cytokines are fundamental in this process. Receiving cytokine-induced messages can result in multiple outcomes including: cellular growth, survival, differentiation or activation. To receive this message the cell must express the cytokines specific receptor(s) and the downstream receptor signaling subunits. Cytokines and their receptors are grouped into specific families based on protein structure. Individual cytokines use distinct receptors for signaling specificity and are usually paired with an additional mutual family receptor subunit. One family of cytokines crucial for the generation and maintenance of cells from both the innate and adaptive immune system are the cytokines that share the common  $\gamma$  chain ( $\gamma$ C) receptor <sup>1</sup>. The  $\gamma$ C family cytokine members include Interleukin-2 (IL-2), IL-4, IL-7, IL-9, IL-15 and IL-21.

Interestingly, a mutation in the  $\gamma$ C subunit has been attributed to one of the most common and most severe immunodeficiency in humans, X-linked severe combined immunodeficiency (X-SCID) <sup>2,3</sup>. Highly publicized and given the moniker “Bubble boy disease”, X-SCID results in the failure to develop both T and NK cells, and have functionally impaired B cells in humans; while X-SCID mice have a major defect in

T, B and NK cells<sup>2-5</sup>. Given that the  $\gamma$ C subunit was identified as one of the receptors for IL-2<sup>6</sup> and a deficiency in IL-2<sup>7-9</sup> did not match the X-SCID phenotype suggested the  $\gamma$ C subunit was crucial for the function of other cytokines involved in T, B and NK cell development and function. As such, IL-4, IL-7, IL-9, IL-15 and IL-21 were identified as the other cytokines utilizing the  $\gamma$ C subunit<sup>3</sup>. The generation of gene-targeted knockout mice revealed both IL-7 (and IL-7R $\alpha$ ) and IL-15 were the actual cytokines that attributed to the X-SCID phenotype<sup>10-13</sup>. Thus, understanding how  $\gamma$ C cytokines and their respective receptors function is essential in the understanding the factors that generate a functional immune system.

## **1.2 The Biology of IL-15.**

### 1.2.1 IL-15 and IL-15 receptors.

IL-15 is a member of the four  $\alpha$ -helix bundle cytokine family<sup>14,15</sup>. First described as a growth factor for T cells with similar function to IL-2, IL-15 has since been shown to mediate diverse biological functions on various immune cells<sup>16</sup>. Furthermore, the expression pattern and regulation of IL-2 and IL-15 are quite different from one another. IL-2 is produced mainly by activated CD4<sup>+</sup> T and CD8<sup>+</sup> T cells through TCR and CD28 stimulation and regulated at the level of mRNA transcription and stabilization<sup>17,18</sup>. Opposite to IL-2, IL-15 mRNA is constitutively expressed at steady-state in multiple cell-types ranging from both hematopoietic (e.g. DCs and monocytes/macrophages) and non-hematopoietic (e.g. fibroblast and epithelial cells) origin. Furthermore, IL-15 mRNA can be enhanced by IFN- $\gamma$  and LPS or upon viral or bacterial stimulation (in DCs and macrophages)<sup>16,19,20</sup>. Nevertheless, the

production IL-15 does not always correlate with mRNA expression and is in fact regulated at multiple levels starting from transcription, translation, intracellular trafficking and secretion <sup>16,20</sup>. Lastly, the receptors of IL-15 are also critical for the secretion of IL-15 (discussed below) <sup>20</sup>. Thus, IL-2 and IL-15 perform their function and are expressed under different physiological and immunological conditions.

IL-15 is produced by IL-15 utilizes three receptors consisting of the unique IL-15R $\alpha$ , the shared IL-2/15R $\beta$  and the common  $\gamma$ C subunits <sup>16</sup>. The IL-15R $\beta$ / $\gamma$  subunits are the major signal transducer for IL-15 activating the Janus kinase (Jak) and signal transducer and activator of transcription (STAT) pathway. In lymphocytes, IL-15R $\beta$  is coupled to Jak1 and STAT3 activation while the  $\gamma$ C associates with Jak3 and STAT5 <sup>16</sup>. IL-15 signaling also triggers other pathways involved in PI3 kinase/AKT, NF- $\kappa$ B, Bcl-2, c-myc and fos/jun activation <sup>16</sup>. Regarding IL-15R $\alpha$ , lymphocytes appear not to signal through this receptor subunit.

IL-15 shares the IL-15R $\beta$ / $\gamma$  subunits with IL-2 and like IL-15, IL-2 also has its own distinct receptor, IL-2R $\alpha$ . Unlike IL-2 and IL-2R $\alpha$ , IL-15 and IL-15R $\alpha$  mRNA is expressed in cells of both hematopoietic and non-hematopoietic origin <sup>16</sup>. Although IL-15 and IL-15R $\alpha$  is widely expressed by diverse cells throughout the body it explicitly affects cells of the innate and adaptive immune system. In particular, a deficiency in this cytokine or its specific receptor, IL-15R $\alpha$ , results in a significant loss of natural killer (NK), invariant NKT (iNKT), memory CD8<sup>+</sup> T cells and intestinal intraepithelial lymphocytes (IEL) <sup>13,21</sup>. In complete contrast, over-expression of IL-



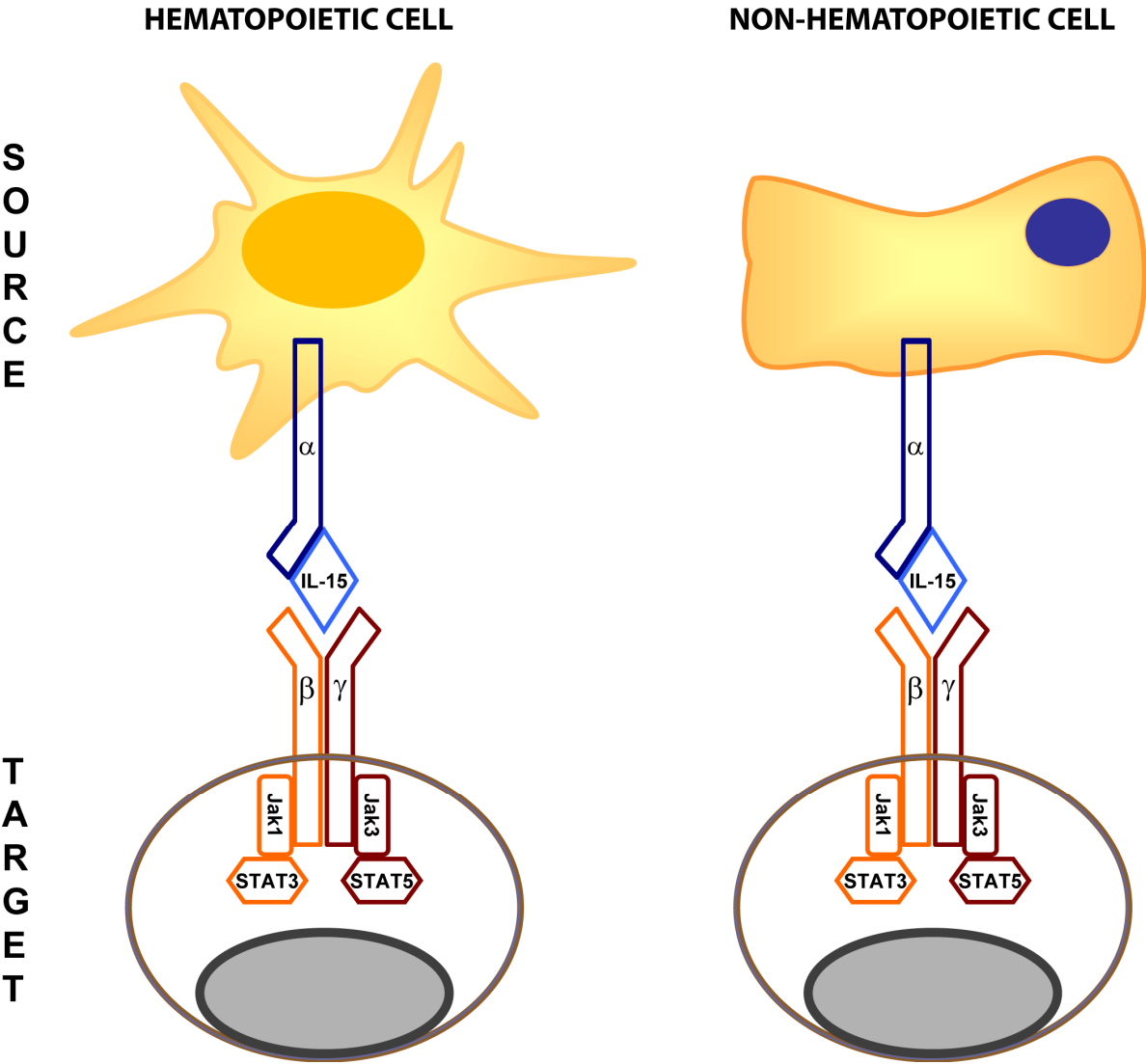
IL-15 in mice leads to the massive expansion of NK and memory CD8<sup>+</sup> T cells and consequently develops fatal lymphocytic leukemia<sup>22</sup>. Therefore, IL-15 signaling must be tightly regulated and distributed conservatively to generate a competent immune system and prevent terminal pathological conditions.

### 1.2.2 Trans-presentation of IL-15.

The striking similarities between IL-15- and IL-15R $\alpha$ -deficient mice were unusual given that IL-15R $\alpha$  has no role in IL-15 signaling. But this similarity suggested an important role for IL-15R $\alpha$  in IL-15 signaling. Unlike conventional cytokines utilizing heterodimeric or –trimeric receptor subunits, IL-15 is thought to work through a unique mechanism of delivery termed “trans-presentation” (depicted in **FIGURE 1**)<sup>23-25</sup>. Trans-presentation involves two cell-types, an IL-15 producing cell (e.g. hematopoietic or non-hematopoietic origin) and an IL-15 responsive cell for this method of cytokine delivery. Specifically, the IL-15 producing cell produces both IL-15 and IL-15R $\alpha$  then shuttles them as a complex to the cell surface to stimulate a neighboring IL-15 responsive cell expressing IL-15R $\beta/\gamma$ C subunits.

Evidence for this novel mechanism of cytokine delivery was derived from both in vitro and in vivo studies. The first suggestion IL-15 was delivered in “trans” was the identification of surface-bound IL-15 on monocytes which stimulated adjacent T cells in vitro<sup>19</sup>. It was further revealed IL-15R $\alpha$  was associated with cell surface IL-15 and this cell surface IL-15 was biologically active<sup>19,26</sup>. Additional evidence supporting the trans-presentation model came when IL-15R $\alpha$ <sup>-/-</sup> CD8<sup>+</sup> T cells

FIGURE 1. Trans-presentation of IL-15.



### **FIGURE 1. Trans-presentation of IL-15.**

Cartoon representing the receptors utilized during IL-15 trans-presentation. The trans-presenting cell (**source**) must express both IL-15 and the specific IL-15R $\alpha$  subunit. Both hematopoietic and non-hematopoietic cells can be the source of IL-15. In order to respond to IL-15, the IL-15-dependent cell (**target**) must express both IL-15R $\beta$  and  $\gamma_c$  subunits in addition to the appropriate intracellular signaling components.

responded to plate-bound IL-15/IL-15R $\alpha$  <sup>27</sup>. Further studies corroborated these in vitro data revealing IL-15R $\alpha$  expression by memory CD8<sup>+</sup> T cells and other IL-15 responsive cells (i.e. iIELs and NK cells) is not required to respond to IL-15 in vivo <sup>28-31</sup>. Transfer experiments further revealed that IL-15R $\beta$ / $\gamma$ C expression is required by NK cells while IL-15R $\alpha$  expression by the host was crucial for in vivo functions of IL-15 <sup>32</sup>. Finally, several studies revealed the same cell producing IL-15 must express IL-15R $\alpha$  to deliver IL-15 <sup>20,28,33</sup>. Overall, both in vitro and in vivo studies supported this mode of cytokine delivery by revealing a) IL-15 and IL-15R $\alpha$  are produced by the same cell to stimulate adjacent cells; b) IL-15R $\alpha$  expression is not required by the responding cell; and c) the responding cell must express the IL-15R $\beta$ / $\gamma$  chain subunits.

These prior studies revealed the basic requirements to receive and respond to IL-15. Because IL-15 and IL-15R $\alpha$  are ubiquitously expressed, it suggests multiple cell-types trans-present IL-15 to NK, iNKT, iIELs and memory CD8<sup>+</sup> T cells. Despite the fact that IL-15 is expressed by multiple sources, it is tightly regulated and competition for IL-15 exist between these responding cells <sup>16,34</sup>. These two basic factors, tight regulation and competition for IL-15, perhaps shape the IL-15 niche for NK, iNKT, iIELs and memory CD8<sup>+</sup> T cells. Since these cells occupy similar anatomical locations, acquiring IL-15 from different sources in these various microenvironments would ensure the generation and maintenance of each population.

Indeed, preferences in cell-specific IL-15 trans-presentation by hematopoietic or non-hematopoietic cells have been shown for memory CD8<sup>+</sup> T cells and intestinal intraepithelial lymphocytes, respectively<sup>28-30</sup>. This cell specificity in IL-15 trans-presentation was not observed for NK and NK1.1<sup>+</sup> T cells as both cellular compartments could partially generate both populations<sup>29</sup>. Recently, we have identified dendritic cells (DC) as one of the main hematopoietic cell-types that support the homeostatic proliferation of memory CD8<sup>+</sup> T cells via IL-15 trans-presentation<sup>35</sup>. For iIELs, we found the expression of IL-15R $\alpha$  solely by intestinal epithelial cells induces the proliferation and survival of iIELs to recovery this population deficient in the IL-15R $\alpha$ <sup>-/-</sup> model<sup>36</sup>. Once again, these studies highlight the fact IL-15R $\alpha$  expression by IL-15-dependent cells is unnecessary but absolutely required on cells in the microenvironment they occupy. Thus, recognizing the cells that trans-present IL-15 will help identify the cellular targets that can directly augment specific IL-15-dependent populations. Despite much progress being made in identifying the trans-presenting cell for memory CD8<sup>+</sup> T cells and iIELs, the specific cell-type(s) trans-presenting IL-15 to NK and iNKT cells is unclear and the function being mediated by IL-15 is also uncertain. Therefore, I have investigated what cellular compartments trans-present IL-15 to NK and iNKT cells. Additionally, I have also identified the tissue-specific functions of IL-15 for NK and iNKT cells (discussed in **Chapter 2 and 3**, respectively).

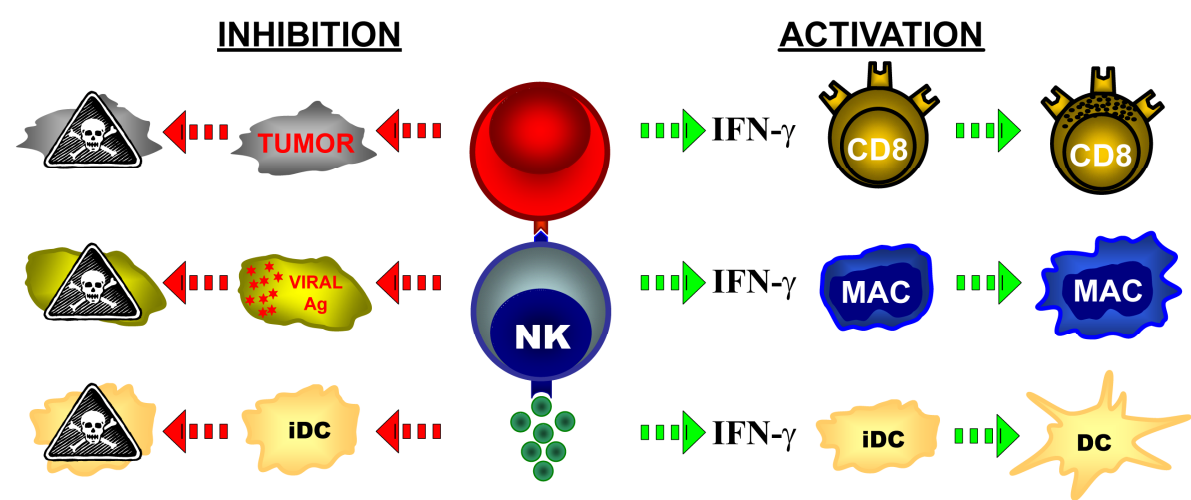
### **1.3 Natural Killer Cells.**

#### 1.3.1 NK cells and their function.

First acknowledged as large granular lymphocytes lacking T and B cell markers, NK cells are now recognized as a critical component of the innate immune system. Unlike the adaptive immune cells, these innate cells respond rapidly to detect and eliminate virus-infected or transformed cells. The name “natural killer” is derived from the initial observation that NK cells could kill tumor cells without prior activation. In more recent years, it has now been accepted these lymphocytes are more than just “natural killers”. In addition to cytolytic activity, NK cells can also release immuno-regulatory cytokines that affect cells from both innate and adaptive immune systems (**FIGURE 2**). These innate lymphocytes are hematopoietic-derived and develop in the bone marrow microenvironment but also occupy other peripheral tissues. In mice, NK cells make up about 0.5 – 1% of the lymphocyte population in the BM, 15-25% in the liver, 1-5% in the spleen, and 10-20% of the lymphocytes in the blood <sup>37</sup>.

These BM-derived innate lymphocytes can act as the first detector and responder in certain immune challenges. NK cells can kill target cells via the granzyme b/perforin exocytosis pathway or by using TRAIL- and FasL-mediated pathways. They can also release cytokines such as IFN- $\gamma$  and TNF- $\alpha$  and chemokines like CCL3, CCL4 and CCL5 to enhance the immune response <sup>38</sup>. This production of cytokines and chemokines can consequently recruit, activate and induce the maturation of DCs, macrophages and CD8<sup>+</sup> T cells. NK cells may also limit the immune response by killing immature DCs and activated CD4<sup>+</sup> T cells <sup>38</sup>. These functional attributes depend heavily on a barrage of germ-line encoded NK cell surface receptors

FIGURE 2. NK cell immune regulation.



## **FIGURE 2. NK cell immune regulation.**

Cartoon illustrating the functional plasticity of NK cells. Detection of transformed- or viral-infected cells or cytokine stimulation results in NK cell activation and subsequent death of the target cell. Activated NK cells can produce various cytokines including IFN- $\gamma$  which can trigger an immune response by inducing the maturation of DCs and macrophages as well as activating CD8<sup>+</sup> T cells. Activated NK cells can also limit the immune response by killing immature DCs. Thus, NK cells are a fundamental part of the immune response linking both innate and adaptive arms of the immune system.



(**TABLE 2**) and various other extracellular cues (IL-2, IL-12, IL-15, IL-18 and type I IFNs) that induce or enhance their functional capabilities<sup>37,39</sup>.

Although first believed to possess the ability to spontaneously kill without prior activation or sensitization, NK cell function is a developmentally acquired process dependent on the strength of receptor stimulation and cytokine responsiveness<sup>39</sup>. The germ-line encoded receptors responsible for NK cell function are acquired during development and function as either an activating or inhibitory stimulus. In mice, the stimulatory balance is dictated by the heterogeneous expression of a variety of receptors that include the CD94/NKG2 family heterodimers, 2B4, Ly49 family members and other c-lectin molecules such as NKG2D, NK1.1, NKp46, NKR-P1B, NKR-P1A and KLRG1. The ligands for the activating receptors can be induced by cellular stress, DNA damage or infection as is the case for NKG2D ligands RAE1, H60 and MULT1 in mice or encoded by the viral pathogen m157 (i.e. Ly49H ligand) expressed by Murine Cytomegalovirus (MCMV)<sup>40-42</sup>. The ligands for the various inhibitory receptors include MHC class Ia molecules and other self-ligands that dampen or inhibit the activating signals received by various activating NK cell receptors. This response can be lost due to recognition of decreased or complete loss of inhibitory ligands (also known as “missing self”) and the induction and recognition of activating ligands<sup>43</sup>. Consequently, this expression and detection system allows NK cells to be equipped and enabled to recognize healthy versus diseased cells, a process known as NK cell “education”.

**TABLE 1. NK cell receptors.**

<b>Receptor</b>	<b>Function</b>	<b>Stage Acquired</b>	<b>Ligand(s)</b>
<b>NKG2D</b>	Activating	Early Immature	Rae1, H60, MULT1
<b>CD94/NKG2A</b>	Inhibitory	Early Immature	Qa1 <sup>b</sup>
<b>CD94/NKG2C</b>	Activating	Early Immature	Qa1 <sup>b</sup>
<b>NK1.1</b>	Activating	Early Immature	?
<b>NKp46</b>	Activating	Early Immature	?
<b>CD16</b>	Activating	Early Immature	IgG
<b>2B4</b>	A/I	Early Immature	CD48
<b>Ly49A</b>	Inhibitory	Late Immature	H2-D <sup>d</sup> , H2-D <sup>k</sup>
<b>Ly49C</b>	Inhibitory	Late Immature	H2-K <sup>b</sup> , H2-K <sup>k</sup> , H2-D <sup>d</sup> , H2-D <sup>k</sup>
<b>Ly49G</b>	Inhibitory	Late Immature	H2-D <sup>d</sup>
<b>Ly49I</b>	Inhibitory	Late Immature	H2-D <sup>k</sup>
<b>Ly49D</b>	Activating	Late Immature	H2-D <sup>d</sup>
<b>Ly49H</b>	Activating	Late Immature	MCMV m157
<b>KLRG1</b>	Inhibitory	Late Mature	E-, N-, R-cadherin

**TABLE 1. NK cell receptors.**

A brief list of murine NK cell activating and inhibitory receptors and the known ligands they interact with is displayed. The receptors are listed in the order each are acquired during development with early immature and late immature being distinguished by Ly49 expression, and late mature is a population of NK cells expressing high levels of CD11b. The stages of NK cell development is shown in

**FIGURE 3.**

Unlike T and B cell education that involves a selection process, NK cell education (also referred to as “licensing” or “disarming”) is a developmental process that generates functionally mature albeit self-tolerant NK cells <sup>44,45</sup>. NK cell education is achieved upon interactions between NK MHC class I inhibitory receptors (Ly49) and corresponding classical MHC class Ia ligands <sup>46</sup>. These inhibitory receptors set the threshold for NK cells responses to target cells under cellular stress or pathogenic invasion. Without this form of instruction, NK cells do not respond to stress- or pathogen-induced activating ligands <sup>44,45</sup>. Hence, NK cell education grants NK cells the ability to become functionally responsive to activating signals and “missing self”. There are situations where both activating and inhibitory ligands are present on the target cell. But under this scenario the balance is geared to either activation status or inhibition based on the strength of the stimulatory signal. Thus, the acquisition of these inhibitory Ly49 molecules and subsequent “education” is crucial for the function of NK cells. Nevertheless, the stage of development where NK cell “education” occurs is unclear and it is thought to require an additional unknown activating signal <sup>33,46</sup>.

### 1.3.2 NK cell development and homeostasis.

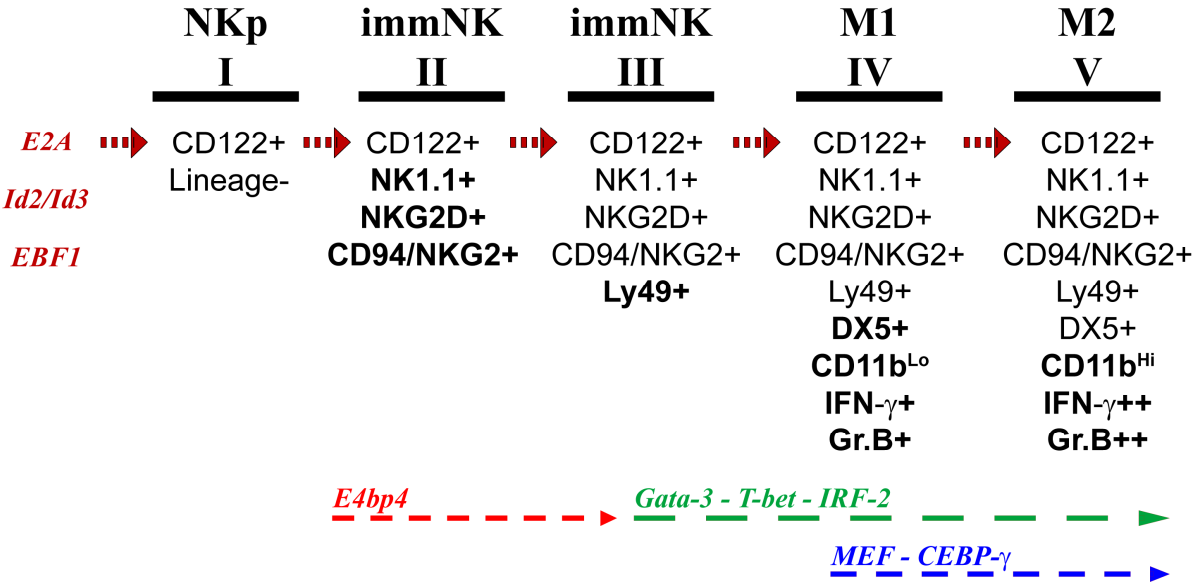
Multiple groups have investigated the lineage commitment of hematopoietic stem cells into NK cells. This commitment to a specific lymphocyte population involves the transition through several developmental stages for functional maturation <sup>47,48</sup>. These events are regulated by various transcription factors and cytokines which dictate the changes in cellular phenotype and function <sup>37</sup>. Until recently, it was

thought that all murine NK cells develop in the BM; however, recent reports have shown a population of NK cells that develop in the thymus<sup>49</sup>. Below I will give a brief overview of NK cell differentiation, and the transcription factors and cytokines involved in the commitment and development of BM-derived NK cells.

The earliest stage of NK cell commitment is the transition of common lymphoid progenitors (CLP) that are defined as Lineage<sup>-</sup> IL-7R $\alpha$ <sup>+</sup> Thy-1<sup>-</sup> Sca-1<sup>Low</sup> c-Kit<sup>Low</sup> to NK precursors (NKp)<sup>30</sup>. NKp's express IL-15R $\beta$  (CD122) and are completely negative for other known NK cell markers, such as NKR-P1C (NK1.1, expressed in C57BL/6 mice) and the various activating and inhibitory receptors<sup>47,48</sup>. Transition from NKp to immature NK cells is marked by the expression of NKG2D, NK1.1 and the CD94/NKG2 heterodimeric complex. This is followed by the stochastic expression of various activating and inhibitory Ly49 receptors; however, these cells are still considered immature<sup>47</sup>. The conversion from immature to mature NK cell is distinguished by the expression of CD49b (DX5) and by the capacity to kill and produce pro-inflammatory cytokines<sup>50</sup>. These functional attributes are enhanced with the up-regulation of CD11b and CD43<sup>47</sup>. Recently, the expression CD27 and CD11b have also been used to discern immature and mature NK cell<sup>51</sup>; however, the differentiation events stringently described above are depicted in **FIGURE 3**.

During the life span of NK cells, from NK commitment to functional maturation, multiple transcription factors are expressed in a developmental manner (discussed below). Furthermore, NK cells continuously express CD122 throughout development and inevitably depend on the cytokine, IL-15, for their generation and

FIGURE 3. NK cell development.



### **FIGURE 3. NK cell development.**

Detailed illustration of BM-derived NK cell differentiation. NK precursors (NKp), stage 1, are the first noted population committed to the NK cell lineage displaying a CD122<sup>+</sup> lineage-negative phenotype. NKp's then differentiate into immature NK cells and acquire various NK cell receptors (**Bold font**) as maturation proceeds. This immature population is further broken down into early (stage II) and late (stage III) immature NK cells and is distinguished from each other by the acquisition of the Ly49 repertoire. DX5 expression marks the transition into mature NK cells and is accompanied by effector function. Mature NK cells gain enhanced functional attributes as terminal maturation proceeds and this event is demarcated by the up-regulation of CD11b. Additionally, various transcription factors that are involved in the NK cell commitment and development are also depicted in this figure.

maintenance (discussed in the next section). Within this differentiation process, there exist other developmental events such as NK cell education (self versus non-self recognition), proliferation and survival that have yet to be definitively resolved.

In the BM, CLPs can give rise to either B cells or NK cells. The commitment to one population over the other appears to be dependent on the E protein transcription factors, E2A; inhibitors of E protein transcription factors, Id2 and Id3; and EBF1 (Id2 and Id3 antagonist) <sup>52,53</sup>. E2A regulates the expression of Id2, Id3 and EBF1. Accordingly, if EBF1 inhibits Id2 and Id3, CLPs will become committed to the B cell lineage. Conversely, if Id2 and Id3 can inhibit E2A expression and subsequently, EBF1 expression, CLPs will become committed to the NK cell lineage. What dictates one fate over the other is currently unknown but is thought to be determined by the excess of transcriptional activity and the levels of each these transcription factors expressed <sup>52</sup>. Interestingly, the inhibition of E2A does not reduce the expression of Id2 and Id3. In fact, Id2 mRNA increases as cells become more restricted to the NK lineage and lineage commitment is noted by the up-regulation of CD122, which Id2 has been shown to regulate <sup>53</sup>. Mice deficient for Id2 lack immature and mature NK cells but not NK precursors <sup>53</sup>. This discrepancy in NK cell commitment may be because of other transcription factors regulating CD122 expression. In Id2<sup>-/-</sup> mice, Id3 is still expressed and may be compensating for the loss of Id2. Ikaros and Runx3 have also been shown to regulate CD122 expression <sup>54,55</sup>. Perhaps this redundancy is to ensure the commitment of CLPs into NKps.



Once committed to the NK cell lineage, multiple transcription factors are involved in the maintenance and functional maturation of NK cells. It has recently been shown mice deficient in E4bp4, a bZIP transcription factor have a specific defect in immature and mature NK cells<sup>56,57</sup>. E4bp4<sup>-/-</sup> NK cells also display a defect in cytolytic activity and cytokine production. Moreover, E4bp4 was shown to regulate Id2 expression but these mice do retain NKp<sup>56</sup>. So E4bp4 likely assists in the differentiation of NKp to immature NK cells. The transition of immature NK cells to mature NK cells appears to involve multiple transcription factors that may act in successive manner.

The generation of mature peripheral NK cells appears to be dependent on Gata-3, T-bet, and IRF-2. Mice deficient in Gata-3<sup>-/-</sup> have a selective loss of mature hepatic NK cells, reduced Ly49, IFN- $\gamma$  and T-bet expression<sup>58</sup>. T-bet<sup>-/-</sup> mice have a severe reduction in all peripheral mature NK cells while IRF-2 affects mature splenic NK cells and NK cell cytotoxicity<sup>59,60</sup>. This defect in function in Gata-3 and IRF-2 deficient mice may be indirect since other transcription factors, such as MEF and CEBP- $\gamma$ <sup>61,62</sup>, contribute to NK cell cytotoxicity and cytokine production but have no role in NK cell numbers or maturation. It could be speculated that E4bp4 regulates Gata-3 which in turn regulate T-bet and IRF-2. These two latter molecules may regulate MEF or CEBP- $\gamma$  to control NK cell function. Undoubtedly, other transcription factors may contribute to the generation and functional maturation of NK cells but the overall molecular events that are involved appear to be developmentally and sequentially regulated. Therefore, understanding how these

molecular events occur will provide an understanding of why and how this heterogeneous lymphocyte population exist and functions.

### 1.3.3 The role of IL-15 in NK cell biology.

The acquisition of IL-15R $\beta$  in the early phase of NK cell development highlights the critical role for IL-15 during the developmental life span of this population. The absence of IL-15 or any of its receptor subunits results in a deficiency of NK cells, which highlights the importance of IL-15 in the overall generation of NK cells<sup>5,13,21,63</sup>. Since trans-presentation is established as a major mechanism of IL-15 delivery, then identifying the "trans-presenting" cell type(s) will enhance our understanding of how IL-15 responses are regulated during NK cell development.

Because radiation-resistant BM stromal cells express IL-15 transcripts, it has long been suggested these cells are a major cell type providing IL-15 to developing NK cells<sup>64-66</sup>. More recently, the utilization of IL-15R $\alpha$  BM chimeras revealed IL-15 and IL-15R $\alpha$ <sup>+</sup> expression by hematopoietic cells rather than non-hematopoietic cells appeared to be a more vital source of IL-15 during the generation of NK cells<sup>29</sup>; however, both cell types can partially support some NK cell development. More specifically, IL-15R $\alpha$  expression by hematopoietic cells has also been shown to regulate Ly49 expression on NK cells thereby implementing IL-15 in the differentiation process<sup>67</sup>. Although it was suggested IL-15R $\alpha$  expression specifically by NK cells affects Ly49 expression<sup>67</sup>, these data still support a role for IL-15 in the acquisition of the Ly49 repertoire.

In addition to its role in differentiation, IL-15R $\alpha$  expression by hematopoietic cells also plays a major role in the homeostatic maintenance of mature NK cells<sup>28,31</sup>. Such maintenance is mediated by IL-15-driven expression of anti-apoptotic molecules Bcl-2 and Mcl-1 and the down-regulation of proapoptotic molecules, Bim and Noxa<sup>32,68-72</sup>. While IL-15R $\alpha$  expression by NK cells appeared to be critical for Ly49 expression, IL-15R $\alpha$  expression by NK cells is not required for its homeostatic proliferation or survival<sup>67</sup>. Hence, IL-15 trans-presentation emerges as the major mode of action during homeostasis<sup>28,31,32,67</sup>. Finally, IL-15 trans-presentation, specifically by dendritic cells (DC) activates NK cells during an immune response to viruses or bacteria<sup>73</sup>. Thus, IL-15 is critical in all aspects of NK cell biology from development to functional maturation to activation.

Overall, these findings suggest that different cell compartments may have distinct roles in NK cell development. So now the initiative must be made to distinguish which hematopoietic and non-hematopoietic cells drive NK cell development and homeostasis through IL-15 trans-presentation. Whether DCs can also trans-present IL-15 during the development and homeostasis of NK cells is unclear. DCs express both IL-15 and IL-15R $\alpha$  and can interact with NK cells in vivo<sup>20,74,75</sup>. Thus, DCs may likely be one of the major hematopoietic cell-types providing IL-15 to NK cells during development and maintenance<sup>76</sup>. Furthermore, since developmental intermediates are found in the spleen and liver, it is possible that the cell-type trans-presenting IL-15 is tissue site specific and/or stage specific.

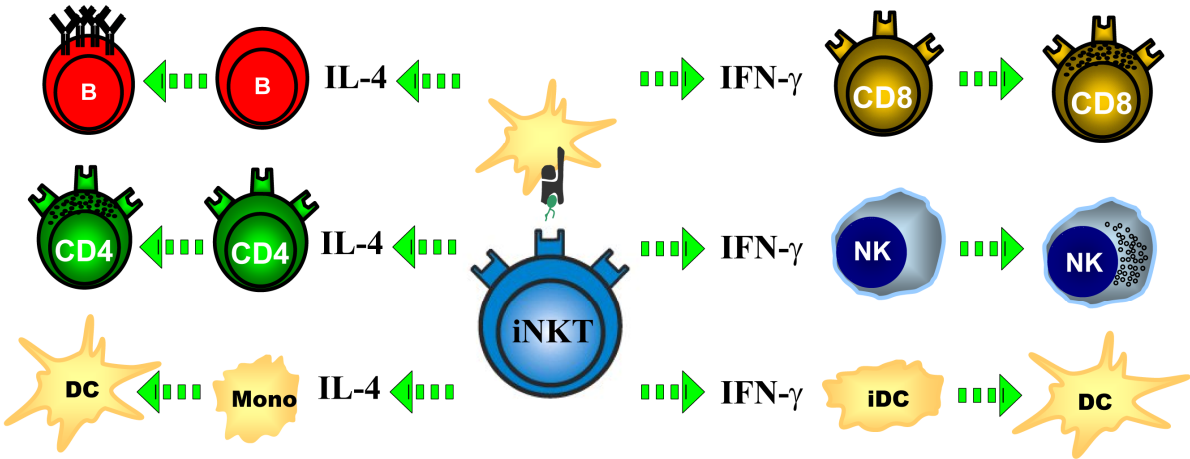
## 1.4 Invariant Natural Killer T Cells.

### 1.4.1 iNKT cells and their function.

Murine NKT cells are a population of T cells that are dependent and selected by CD1d, a MHC class I-like molecule that presents glycolipid antigen<sup>77-80</sup>. Within this CD1d-dependent population of lymphocytes there exist two types of NKT cells that are classified on the basis of T cell receptor (TCR) usage. Type I NKT cells express an invariant TCR V $\alpha$ 14-J $\alpha$ 18  $\alpha$  chain coupled with either V $\beta$ 8, V $\beta$ 7 or V $\beta$ 2  $\beta$  chain (V $\alpha$ 24-J $\alpha$ 18/V $\beta$ 11 in humans) and are also referred to as invariant NKT (iNKT) cells<sup>81-83</sup>. iNKT cells are reactive to the glycolipid,  $\alpha$ -Galactosylceramide ( $\alpha$ GalCer) and also recognize the self-ligand isoglobotrihexosylceramide<sup>84,85</sup>. This recognition of  $\alpha$ GalCer allows iNKT cells to be detected by  $\alpha$ GalCer-loaded CD1d-tetramers<sup>86,87</sup>. Unlike iNKT cells, type II NKT cells express a diverse repertoire of TCR  $\alpha$  chains and are also non-reactive to  $\alpha$ GalCer thus undetectable by CD1d-tetramers<sup>88</sup>. Herein, I will discuss what is known and unknown about iNKT cell biology.

In addition to the invariant TCR, iNKT cells express multiple cell surface receptors associated with NK cells. Consequently, they were given the moniker “natural killer” T cells; however, not all iNKT cells express NK cell receptors (discussed in the next section). But similar to NK cells, iNKT cells are functionally active immediately upon stimulation and thus behave in an innate-like fashion<sup>89</sup>. One innate attribute is the ability to activate cells from both the innate and adaptive immune system (**FIGURE 4**). This immune regulation is because iNKT cells can produce multiple cytokines such as IL-4, IFN- $\gamma$  and IL-17 which are usually restricted to specific

FIGURE 4. iNKT cell immune regulation.



**FIGURE 4. iNKT cell immune regulation.**

Cartoon illustrating the immuno-regulatory properties of iNKT cells. Activation of iNKT cells can induce the production of both Th1 (IFN- $\gamma$ ) and Th2 (IL-4) cytokines by iNKT cells. The production of IFN- $\gamma$  by iNKT cells can activate both NK and CD8<sup>+</sup> T cells and can also induce the maturation of DCs. The Th2 cytokines being produced by iNKT cells has been shown to stimulate the differentiation of monocytes into DCs as well as mount a humoral immune response. Thus, the activation of iNKT cells and subsequent cytokines they produce can be the determining factors in dictating the outcome of the immune response.

subsets of conventional CD4<sup>+</sup> T cells<sup>90-92</sup>. Consequently, the ability to rapidly produce multiple cytokines can dictate the immune response and subsequent outcome.

While iNKT cells are known to produce multiple cytokines it is now appreciated that multiple factors contribute to the cytokines being produced. One such factor is that subsets of iNKT cells exist in both mice (CD4<sup>+</sup> and CD4<sup>-</sup>CD8<sup>-</sup> also called double negative [DN] subsets) and humans (CD8<sup>+</sup>, CD4<sup>+</sup> and DN subsets). In humans and in some cases in mice, certain iNKT subsets can favor a Th2 cytokine profile (CD4<sup>+</sup> iNKT cells) while other subsets display a Th1 or Th17 cytokine profile (DN iNKT cells)<sup>90,92-94</sup>. In addition, the strength of TCR stimulation can affect IL-4 versus IFN- $\gamma$  production. Whereas a quick TCR stimulation induces more IL-4, a longer interaction between CD1d-TCR invokes a strong IFN- $\gamma$  response<sup>95,96</sup>. Other factors influencing the cytokine production include the glycolipid activating iNKT cells. The glycolipid,  $\alpha$ GalCer, is known to induce some IL-4 and copious amounts of IFN- $\gamma$ . OCH, a modified form of  $\alpha$ GalCer, invokes a remarkably different response by inducing more IL-4 and lower amounts of IFN- $\gamma$ <sup>97-99</sup>. Hence, the cytokine(s) production by activated iNKT cells is a dynamic event which can determine the immune response. Therefore, understanding all the factors contributing to the function of iNKT cells is essential and exploitable knowledge.

Interestingly, the immuno-regulatory activity of iNKT cells includes a protective role in various autoimmune diseases and a pathogenic role in various inflammatory

diseases<sup>100,101</sup>. This protective and pathogenic role of iNKT cells is also observed in various tumor models. For instance, several independent studies have shown iNKT cells can enhance or suppress an anti-tumor response<sup>102-104</sup>. Initially, murine iNKT cells were shown to have anti-tumor function by rejecting tumors and preventing tumor metastasis<sup>105,106</sup>. These effects were mediated by iNKT cell production of IFN- $\gamma$  and subsequent activation of DCs, NK, B and CD8<sup>+</sup> T cells. Conversely, the production of IL-4 and IL-13 by iNKT cells contribute to the suppressive activity observed in various murine tumor models<sup>102,104</sup>. Taken together, these studies reveal iNKT activation can be either beneficial or detrimental to the host depending on the antigen or subset of cells being activated. The basis of these paradoxical features appears to depend on either the tissue site in which the iNKT subset is isolated or perhaps developmental signals they receive in these tissue microenvironments. For these reasons, understanding the events that regulate the development of iNKT cells is essential for host immunity and possible clinical exploitation.

#### 1.4.2 iNKT cell development and homeostasis.

Similar to conventional T cells, iNKT cell are derived from the same hematopoietic precursor where they also develop in the thymus making up ~0.5 - 1% of all lymphocytes residing in the thymus<sup>89</sup>. Additionally, iNKT cells like conventional T cells are also found in the periphery where they represent approximately 2% of all lymphocytes in the spleen and up to ~20 - 30% of the lymphocytes in the liver<sup>89</sup>. However, the developmental process of iNKT cells does not follow the conventional



T cell pathway<sup>79,90,107</sup>. Developing iNKT cells go through multiple transitional stages that can be followed by staining with CD1d-tetramers along with other cell surface markers (such as CD4, CD44, and NK1.1)<sup>86,87</sup> (**FIGURE 5**).

The earliest detectable stage of iNKT cell development occurs after V $\alpha$ 14-J $\alpha$ 18 rearrangement and positive selection by CD1d<sup>+</sup> double positive (CD4<sup>+</sup>CD8<sup>+</sup>, DP) thymocytes<sup>80</sup>. This population is detectable by CD1d-tetramers and exhibits a CD4<sup>+</sup>CD44<sup>Low</sup>NK1.1<sup>-</sup> cellular phenotype<sup>86,90</sup>. As previously mentioned, DN iNKT subsets also exist in mice but the stage where CD4 is down-regulated giving rise to the DN population is currently an ambiguous developmental event. Despite being immature, CD4<sup>+</sup>CD44<sup>Low</sup>NK1.1<sup>-</sup> iNKT cells are capable of producing IL-4 when stimulated in vitro<sup>90,107</sup>. These immature iNKT cells expand and differentiate into CD44<sup>High</sup> cells which are capable of producing both IL-4 and a small amount IFN- $\gamma$  (in vitro stimulation) and exiting the thymus to seed the periphery<sup>90,107</sup>. Although thymic iNKT cell emigration occurs at this stage, a portion of iNKT cells begin to up-regulate the chemokine receptor, CXCR3, which assists in the retention of thymic iNKT cells<sup>108</sup>. The final stage of development or maturation is demarcated by NK1.1 and CD122 expression and is accompanied by further expansion<sup>59,90</sup>. Unlike their predecessors, mature iNKT cells produce more IFN- $\gamma$  when activated in vitro, but retain some ability to produce IL-4<sup>90</sup>. Again, atypical of normal conventional T cell development, this final stage of maturation of iNKT cells can occur either in the thymus or the periphery.

## THYMUS



## **FIGURE 5. iNKT cell development.**

Detailed illustration of iNKT cell differentiation occurring in the thymus and periphery. The first stage of development is an immature cell bearing  $CD44^{Low}NK1.1^{-}$  cell surface phenotype and is capable of producing IL-4. This immature population can expand and differentiate into a population expressing  $CD44^{High}NK1.1^{-}$  phenotype. At this stage this population is still capable of producing IL-4 and can emigrate from the thymus to seed the periphery. In the thymus and periphery, iNKT cells displaying  $CD44^{High}NK1.1^{-}$  cell surface markers can expand and differentiate into a population also expressing  $NK1.1^{+}$ , which has previously been described as a mature population of iNKT cells. This population of iNKT cells is geared more towards producing IFN- $\gamma$  but is still capable of producing IL-4. In addition to cell surface receptors acquired throughout development, various transcription factors that are involved in iNKT cell commitment and development are also depicted in this figure.

While maturation of iNKT cells is usually defined by the acquisition of NK1.1 expression, functional maturation does not always correlate with NK1.1 expression. Despite the expression of NK1.1, thymic iNKT cells in normal mice do not express IFN- $\gamma$  in response to  $\alpha$ GalCer<sup>87</sup> suggesting these cells are suppressed, or not inadequately stimulated in the thymus, or perhaps not truly functionally mature. Moreover, it has recently been suggested that the NK1.1<sup>-</sup> iNKT cells in the periphery may be mature or possibly a subset of iNKT cells that only transiently expresses NK1.1<sup>109</sup>. Therefore, functional responsiveness, or true maturation of iNKT cells is not inherent to all NK1.1<sup>+</sup> iNKT cells and therefore is likely subject to additional regulation. Regardless of the phenotype or state of functional maturation, peripheral iNKT cells are maintained in the periphery by prosurvival factors, Bcl-xL and Bcl-2, and undergo slow homeostatic proliferation<sup>110</sup>. Such maintenance is dependent on IL-15 (discussed in the next section) and independent of CD1d-TCR interaction<sup>34,111</sup>. However, the cell-type providing IL-15 during peripheral homeostasis is currently unknown.

Throughout iNKT cell development, a transcriptional network exists that allows for the generation, differentiation and functional maturation of iNKT cells. At the earliest point of iNKT cell development various transcription factors are critical in the commitment to the iNKT lineage by regulating TCR rearrangement. For example, a complete loss of iNKT cells is apparent in mice deficient in HEB (E protein transcription factor), ROR $\gamma$ t and the Runx1 (Runx family member)<sup>91,112,113</sup>. The complete loss of iNKT cells in HEB<sup>-/-</sup> and ROR $\gamma$ t<sup>-/-</sup> mice is due to the lack of V $\alpha$ 14-

J $\alpha$ 18 rearrangement; however, V $\alpha$ 14-J $\alpha$ 18 rearrangement does occur in Runx1<sup>-/-</sup> mice<sup>112,113</sup>. This suggests HEB and ROR $\gamma$ t act upstream of Runx1 to generate the invariant TCR while Runx1 is critical either during or immediately after positive selection by CD1d<sup>+</sup> DP thymocytes. Additionally, ROR $\gamma$ t expression appears to be critical for IL-17 production by iNKT cells, revealing multiple steps of developmental regulation being carried out by various transcriptional factors<sup>91</sup>.

Once iNKT cells are selected, the CD4<sup>+</sup> CD44<sup>Low</sup> NK1.1<sup>-</sup> iNKT cells begin to express several transcription factors that are essential in granting early functional features (IL-4 production) and initiating cellular differentiation. Specifically, these immature CD4<sup>+</sup> CD44<sup>Low</sup> NK1.1<sup>-</sup> iNKT cells begin exhibiting functional capabilities that are defined by the ability to produce Th2 cytokines upon stimulation<sup>90</sup>. Both PLZF (BTB-ZF family transcription factor) and Gata-3 have been shown to contribute to this early functional attribute as shown in gene-targeted deficient mice<sup>114,115</sup>. In addition to containing low numbers of iNKT cells, iNKT cells from PLZF<sup>-/-</sup> mice display a CD4<sup>+</sup> CD44<sup>Low</sup> NK1.1<sup>-</sup> phenotype and produce low levels of IL-4 upon activation<sup>115</sup>. Likewise, a deficiency in Gata-3 also results in a defect in both IL-4 and IL-13 production by iNKT cells<sup>114</sup>. Furthermore, Gata-3<sup>-/-</sup> mice also have a severe reduction in peripheral iNKT cells. But unlike PLZF<sup>-/-</sup> iNKT cells, Gata-3<sup>-/-</sup> iNKT cells display a mature NK1.1<sup>+</sup> phenotype<sup>114</sup>. It is uncertain if PLZF directly regulates Gata-3 as Gata-3 expression was not analyzed in PLZF<sup>-/-</sup> iNKT cells. Nevertheless, it is clear both are crucial for the early developmental events in iNKT cell biology.

The last stage of differentiation, which includes the up-regulation of NK1.1 and the ability to express IFN- $\gamma$ , is dependent on T-bet expression<sup>59</sup>. Specifically, T-bet expression by iNKT cells regulates various molecules including NK1.1, CXCR3, and CD122 expression. These cell surface molecules are crucial for various iNKT cell developmental events including thymic retention (CXCR3) and responsiveness to IL-15 (CD122). Moreover, T-bet also regulates various functional proteins including IFN- $\gamma$ , granzyme B and perforin<sup>59,116</sup>. As expected, T-bet<sup>-/-</sup> mice severely lack thymic and peripheral iNKT cells particularly the NK1.1<sup>+</sup> iNKT cell population<sup>116</sup>. Regarding cytokine production, T-bet<sup>-/-</sup> iNKT cells have a deficiency in IFN- $\gamma$  production but still retain the capacity to produce IL-4 upon stimulation<sup>59,116</sup>. Thus, T-bet is crucial in the transition from CD44<sup>High</sup> NK1.1<sup>-</sup> cells to CD44<sup>High</sup> NK1.1<sup>+</sup> cells. In addition, it also endows mature iNKT cells with the ability to respond to IL-15 and the capability to produce IFN- $\gamma$ . Although it is clear that T-bet is important for iNKT maturation, how T-bet is regulated in developing iNKT cells in vivo is not known. Together, these studies suggest that a complex network of transcription factors cooperate with each other to generate and maintain this unique functional T cell population.

#### 1.4.3 The role of IL-15 in iNKT cell biology.

Like other IL-15-dependent lymphocytes, iNKT cells are severely reduced in mice deficient in IL-15 or any of its receptors<sup>5,13,21,63</sup>. Specifically, IL-15<sup>-/-</sup> mice lack normal numbers of thymic and peripheral iNKT cells<sup>13,34</sup>. In these mice, all differentiation stages are present; however, CD44<sup>High</sup> NK1.1<sup>+</sup> cells are preferentially

lost. This is likely due to an increased sensitivity to IL-15 as CD44<sup>High</sup> NK1.1<sup>+</sup> cells have higher expression of CD122 compared to the immature populations<sup>34,116</sup>. In addition to its role in development, IL-15 is also important for iNKT cell homeostasis. Adoptive transfer experiments revealed iNKT cells do not undergo homeostatic proliferation or survive in IL-15-deficient mice<sup>34,111</sup>. So similar to NK cells, IL-15 appears to be required late during iNKT cell development and homeostasis particularly, as a survival and expansion factor.

As mentioned in the prior sections, trans-presentation is the major mechanism for IL-15 delivery but the cell-type(s) trans-presenting IL-15 to iNKT cells has not been described. Prior studies using various IL-15R $\alpha$  BM chimeras revealed IL-15R $\alpha$  expression by either hematopoietic or non-hematopoietic cells partially recovers peripheral NK1.1<sup>+</sup> T cell numbers<sup>29</sup>. Unfortunately, this study did not specifically investigate effects on iNKT cells as CD1d-tetramers were not used and not all iNKT cells express NK1.1. Moreover, neither the thymus nor the functions of iNKT cells were examined. As such, these results may have described the requirements for IL-15R $\alpha$  by conventional T cells expressing NK1.1 and may not be reflective of true iNKT cells. More recently, NF- $\kappa$ B activation was shown to control IL-15R $\alpha$  expression by iNKT cells and presumed to enhance IL-15 signaling<sup>117</sup>. Taken together, the phenotype of IL-15R $\alpha$ <sup>-/-</sup> mice and the role of NF- $\kappa$ B in IL-15R $\alpha$  expression makes it plausible that iNKT cells may respond to IL-15 through the IL-15R $\alpha\beta\gamma$  heterotrimeric complex and/or IL-15 trans-presentation. Nevertheless, the

requirement of IL-15R $\alpha$  expression by iNKT cells and the cells they interact with is still unclear.

So while IL-15 is clearly critical for the overall numbers of iNKT cells in the thymus and periphery, the source of IL-15 has not been identified. iNKT cells, like NK cells, interact with DCs, a known source of IL-15. Additionally, mice with genetic defects in the NF- $\kappa$ B family member, RelB, lack thymic DCs, medullary thymic epithelial cells and have a severe deficiency in iNKT cells <sup>118-120</sup>. In RelB<sup>-/-</sup> mice, IL-15 mRNA levels are decreased in the thymic stroma suggesting non-hematopoietic cells may provide a source of IL-15 but this has not been analyzed <sup>119</sup>. In the periphery, hepatic stellate cells (non-hematopoietic origin) provide a source of IL-15 and have been shown to maintain hepatic iNKT cells <sup>121</sup>. Whether hematopoietic cells are a source of IL-15 in the liver is unclear; however, the liver microenvironment contains macrophages and DCs which are known IL-15-producing cells <sup>122</sup>. So unlike the other IL-15-dependent populations far less is known about the role of IL-15 trans-presentation during iNKT cell development and homeostasis. Lastly, whether IL-15 contributes to other aspects of iNKT cell biology, such as functional maturation or activation of iNKT cells, has not been investigated.

## **1.5 Statement of Objective.**

### 1.5.1 Known's and unknowns of IL-15 in NK and iNKT cell biology.

Both IL-15<sup>-/-</sup> and IL-15R $\alpha$ <sup>-/-</sup> mice have a deficiency in NK and iNKT cells, which highlights the importance of both IL-15 and IL-15R $\alpha$  in the development and



homeostasis of NK and iNKT cells <sup>13,21</sup>. Intriguingly, both NK and iNKT cell development includes differentiation through multiple intermediate stages, tissue-specificity and subsets with differing functional attributes. After development is complete, homeostatic mechanisms are present that contribute to maintaining normal numbers of NK and iNKT cells in the periphery. Additional studies have also revealed IL-15 involvement in the activation of NK and iNKT cells <sup>73,74</sup>. Thus, proper development and maintenance of NK and iNKT cells is a requisite for the protection from invading pathogens.

Collectively, the past studies suggest the pleiotropic cytokine, IL-15, is a key regulator in NK and iNKT cell ontogeny. Yet, these past studies never addressed how IL-15 can control and discriminate between each event (differentiation, proliferation, survival and activation). Additionally, the cell-types trans-presenting IL-15 to NK and iNKT cells during these events is currently unclear. These issues are further compounded by the fact that the periphery is seeded with a heterogeneous population of immature and mature NK and iNKT cells, and these populations reside together in multiple tissue sites <sup>37,89</sup>. The finding that trans-presentation is a major mechanism of IL-15 delivery has generated great interest in identifying the “trans-presenting” cell type(s) and may provide clues behind the regulation of IL-15. Although progress has been made, the specific identity of these cells has remained elusive. Equally unclear is the expression of IL-15R $\alpha$  on IL-15-responding cell-types as both cells have been shown to express IL-15R $\alpha$ . Nevertheless, the finding that the expression of IL-15R $\alpha$  by multiple IL-15-dependent cells is not required for their generation or maintenance supports the mechanism of IL-15 trans-presentation as

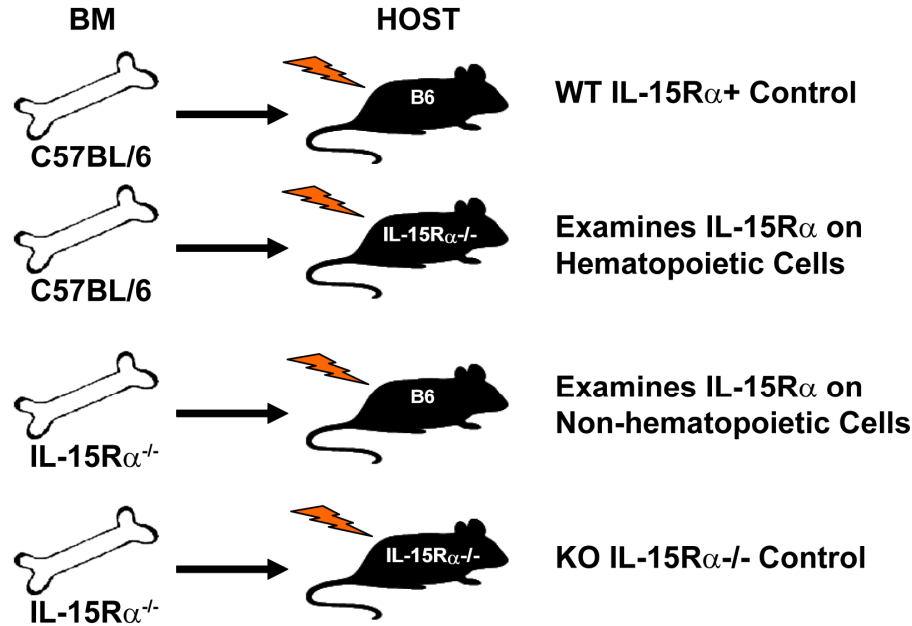
the major source of action<sup>28-31</sup>. Thus, identifying the cell-type trans-presenting IL-15 is essential in understanding NK and iNKT cell biology.

#### 1.5.2 Dissertation Objective and Hypothesis.

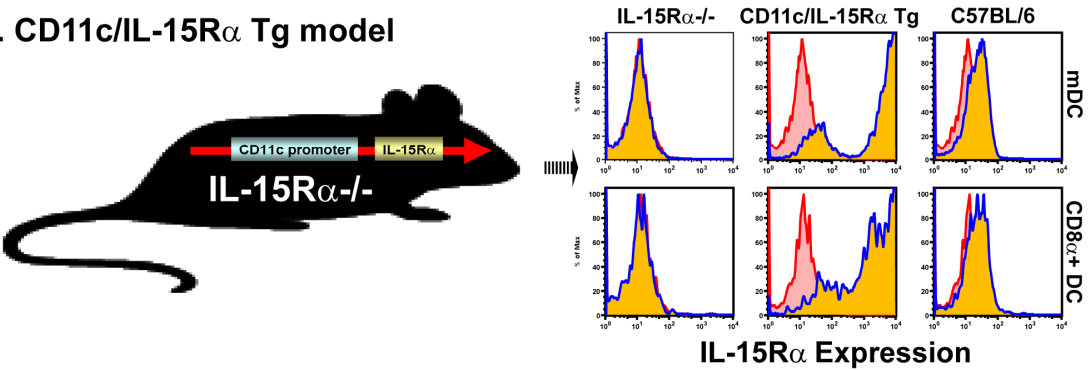
The **overall objective of this investigation** was to determine the cellular function of IL-15 and the potential mediators of IL-15 trans-presentation that control the immunological niche of NK and iNKT cells *in vivo*. To execute this objective, I utilized bone marrow chimeras and transgenic mice (**FIGURE 6**) to dissect what cell-type trans-presents IL-15 to drive the differentiation, functional maturation, proliferation, survival and homeostasis of NK and iNKT cells. The **central hypothesis** of this dissertation is that IL-15 trans-presentation by distinctive IL-15R $\alpha$ -expressing cell-types drives critical immunological events in NK and iNKT cell ontogeny. My specific aims were to: **AIM I:** define the IL-15R $\alpha$ <sup>+</sup> cell-types driving the differentiation; proliferation; functional maturation and homeostasis of NK cells via IL-15 trans-presentation in the various NK cell tissue microenvironments. Specifically, in **1a)** I analyzed the recovery of the various stages of NK cell development when IL-15R $\alpha$  expression is restricted to hematopoietic and non-hematopoietic cells; **1b)** analyzed the role of IL-15R $\alpha$  expression by NK cells in Ly49 expression by generating mixed BM chimeras; and **1c)** assessed the role of DC-mediated IL-15 trans-presentation in NK cell development and homeostasis. In **AIM II:** define the IL-15R $\alpha$ <sup>+</sup> cell-types driving the differentiation; proliferation;

**FIGURE 6. IL-15R $\alpha$  Mouse Models.**

**A. IL-15R $\alpha$  BM Chimeras**



**B. CD11c/IL-15R $\alpha$  Tg model**



## **FIGURE 6. IL-15R $\alpha$ Mouse Models.**

The models used to investigate the cell-type(s) trans-presenting IL-15 to NK and iNKT cells. A) IL-15R $\alpha$  BM chimeras were utilized to restrict IL-15R $\alpha$  expression to either the hematopoietic or non-hematopoietic compartment. B) A mouse model that restricts IL-15R $\alpha$  expression solely to CD11c<sup>High</sup> cells (both described in Chapter 5).

survival; functional maturation and homeostasis of iNKT cells via IL-15 trans-presentation in the various iNKT cell tissue microenvironments. Specifically, in **Ila)** I analyzed the role of hematopoietic and non-hematopoietic cells in generating iNKT cells in the various tissue sites via IL-15 trans-presentation; and in **Ilb)** assessed the role of DC-mediated IL-15 trans-presentation in iNKT cell development and homeostasis. Previous data that support my hypothesis revealed that IL-15 trans-presentation by both hematopoietic and non-hematopoietic cells contribute to the generation of NK and iNKT cells in a tissue-specific fashion <sup>29</sup>. The **general rationale** for my research was to identify the specific cell-types mediating the *in vivo* function of IL-15 and the parameters controlling the specific events in each niche in order to manage pathological and therapeutic conditions.

I tested this hypothesis and accomplished the proposed objective by addressing the following two specific aims:

**Specific Aim 1 (Described in Chapter 2):** I determined the cell-types driving the differentiation, proliferation, functional maturation and homeostasis of NK cells via IL-15 trans-presentation in the various NK cell tissue microenvironments. Specifically, I revealed DCs promote the development and homeostasis of NK cells via IL-15 trans-presentation by using a model wherein CD11c<sup>+</sup> DCs are the sole cell-type trans-presenting IL-15 <sup>35</sup>. By using this model, I provided evidence that DCs contribute to the development and homeostatic maintenance of NK cells via IL-15 trans-presentation. In addition, I have identified specific stages of NK cell

development in which IL-15 trans-presentation functions under steady-state conditions and identified what cellular compartment is trans-presenting IL-15 during these stages. Lastly, I revealed IL-15R $\alpha$  expression by NK cells is unnecessary for Ly49 expression. Overall, an additional role for NK-DC interactions has been identified whereby DCs support the developmental and homeostatic niche of NK cells in a tissue- and stage-specific manner.

**Specific Aim 2 (Described in Chapter 3):** I identified the cell-types driving the differentiation, proliferation, functional maturation and homeostasis of iNKT cells via IL-15 trans-presentation in the various iNKT cell tissue microenvironments. In particular, I revealed the IL-15-driven events in iNKT cell development are mediated by distinct cell types via IL-15 trans-presentation, depending on the tissue microenvironment. In the thymus, radiation-resistant thymic stromal cells provided IL-15 for iNKT cell survival. Whereas in the periphery; both radiation-sensitive and -resistant cells provided IL-15 for survival, proliferation and maturation. DCs, a radio-sensitive cell, were also shown to be critical for the homeostasis of peripheral iNKT cells. Furthermore, IL-15 signaling was shown to impact the functional maturation and activation of hepatic iNKT cells. Overall, I have defined the tissue-specific roles of IL-15 in iNKT cell biology and have identified the specific cells regulating the development, homeostasis, and activation of iNKT cells via IL-15 trans-presentation.

## **CHAPTER 2**

### **The Role of IL-15 Trans-Presentation During the Development and Homeostasis of Natural Killer Cells.**

#### **2.1 Introduction**

NK cells are an intricate part of the innate immune system providing a first line of defense against pathogens and transformed cells. What's more, NK cells can act as trigger to initiate or even boost the adaptive immune response. These features have been exploited in the clinical setting; yet, utilizing their full potential has been a perplexing challenge. To overcome this hurdle the scientific community must understand the biology of NK cells including the regulation of their development, maintenance and effector function.

Given that the generation, maintenance and effector function of NK cells are dependent on IL-15 it is important to understand the basic biology between NK cells and IL-15. Since IL-15 can be delivered via trans-presentation it is essential to identify the source of IL-15 in individual microenvironments harboring NK cells. Identification of these tissue-specific IL-15R $\alpha^+$  cells will provide a wealth of knowledge regarding how the development and maintenance of NK cells are regulated by IL-15.

Currently, the source of IL-15 during NK cell development and homeostasis is unclear. Thus, I have investigated the cell-types driving the differentiation, proliferation, functional maturation and homeostasis of NK cells via IL-15 trans-presentation in the various NK cell tissue microenvironments.

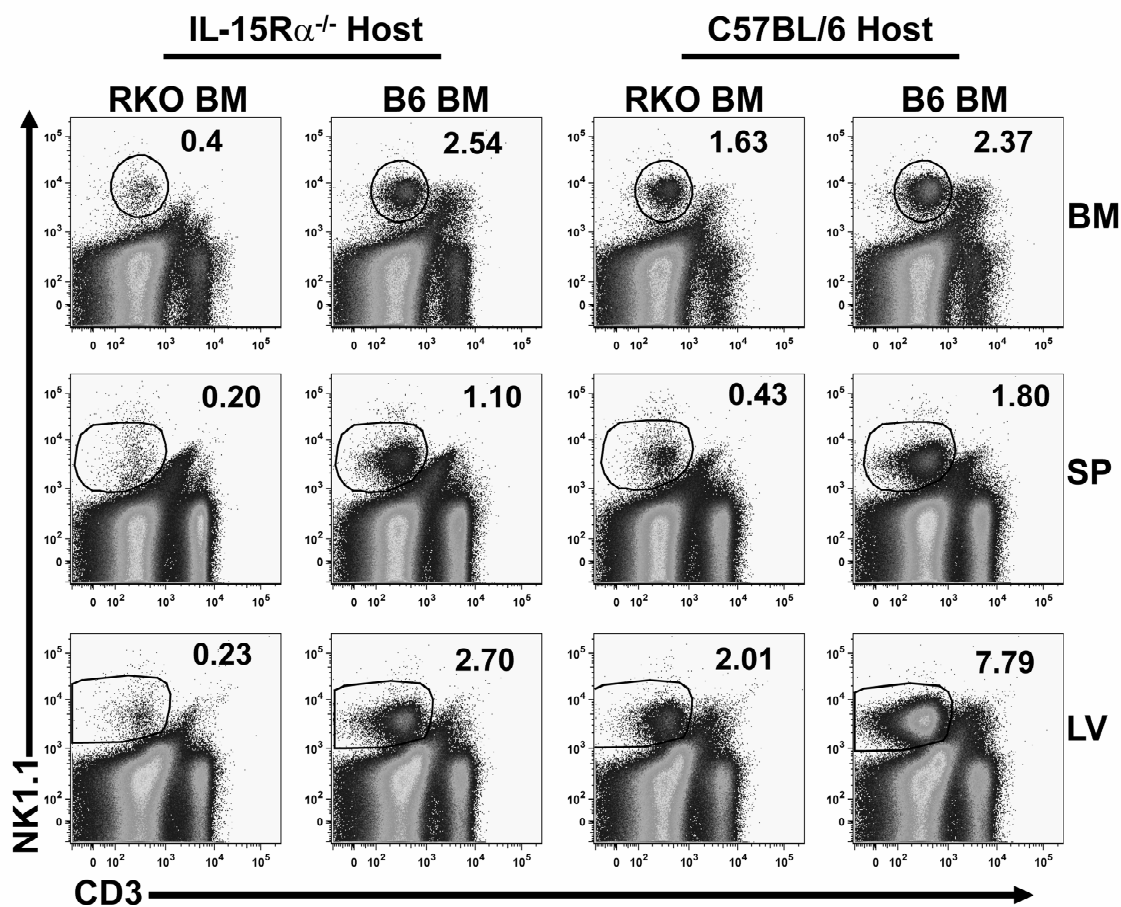
## 2.2 Results

### “ 2.2.1 IL-15R $\alpha$ expression by hematopoietic cells is essential for NK cell development.

In prior BM chimera studies <sup>29,67</sup>, the stages of NK cell development being recovered were not determined. Therefore, we generated various IL-15R $\alpha$  BM chimeras to define the roles of IL-15 trans-presentation by hematopoietic and non-hematopoietic cells during NK cell development. Since NK cells develop in the BM and are abundant in the liver and spleen, these tissues were examined to assess NK cell recovery between the different groups (**FIGURE 7**). Similar to previous reports, the numbers of NK1.1<sup>+</sup> CD3<sup>-</sup> cells in the BM were restored to 75% of control levels in chimeras expressing IL-15R $\alpha$  exclusively by hematopoietic cells (B6 BM  $\rightarrow$  RKO host) (**FIGURE 8**). In the liver and spleen, NK cell generation was recovered to 32% and 50% of control levels, respectively revealing the hematopoietic cells as the dominant cell-type involved in the generation of NK cells (**FIGURE 8**). In comparison, expression of IL-15R $\alpha$  by non-hematopoietic cells (RKO BM  $\rightarrow$  B6 host) also

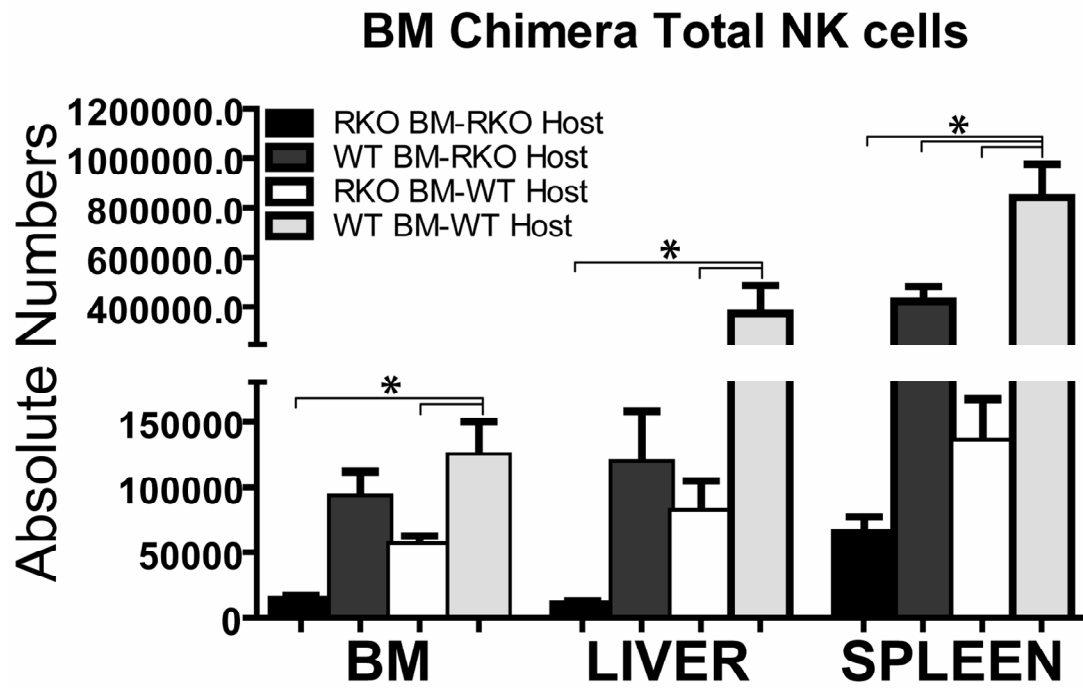


**FIGURE 7. Requirement of IL-15R $\alpha$  by hematopoietic and non-hematopoietic cells in the generation of NK cells.**



**FIGURE 7. Requirement of IL-15R $\alpha$  by hematopoietic and non-hematopoietic cells in the generation of NK cells.** NK1.1<sup>+</sup> CD3<sup>-</sup> cells recovered in the BM, liver and spleen of the various IL-15R $\alpha$  bone marrow chimeras. Data are representative of three independent experiments, n = 8 mice per group.

FIGURE 8. The total number of NK cells recovered in BM chimeras.

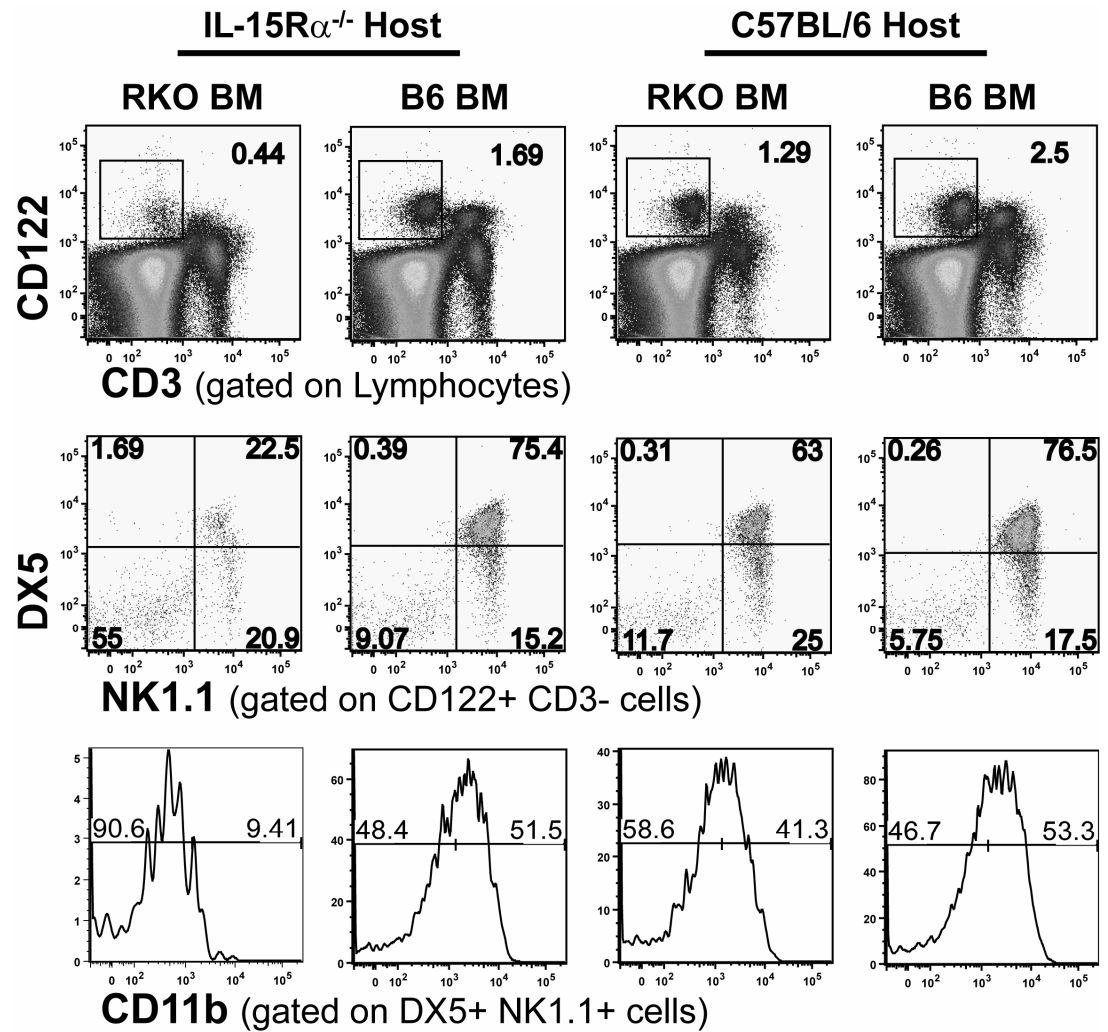


**FIGURE 8. The total number of NK cells recovered in BM chimeras.** Absolute numbers of NK cells (NK1.1<sup>+</sup> CD3<sup>-</sup> cells) in the respective tissues of the various IL-15R $\alpha$  bone marrow chimeras. IL-15R $\alpha$ <sup>-/-</sup> BM into IL-15R $\alpha$ <sup>-/-</sup> host (black bar), WT BM into IL-15R $\alpha$ <sup>-/-</sup> host (dark gray bar), IL-15R $\alpha$ <sup>-/-</sup> BM into WT host (white bar), and WT BM into WT host (light gray bar). The absolute numbers were average of 3 independent experiments, n= 8 mice per group. Error bars represent S.E., \* p  $\leq$  0.05.

generated NK cells albeit less significant than the hematopoietic compartment (**FIGURE 8**).

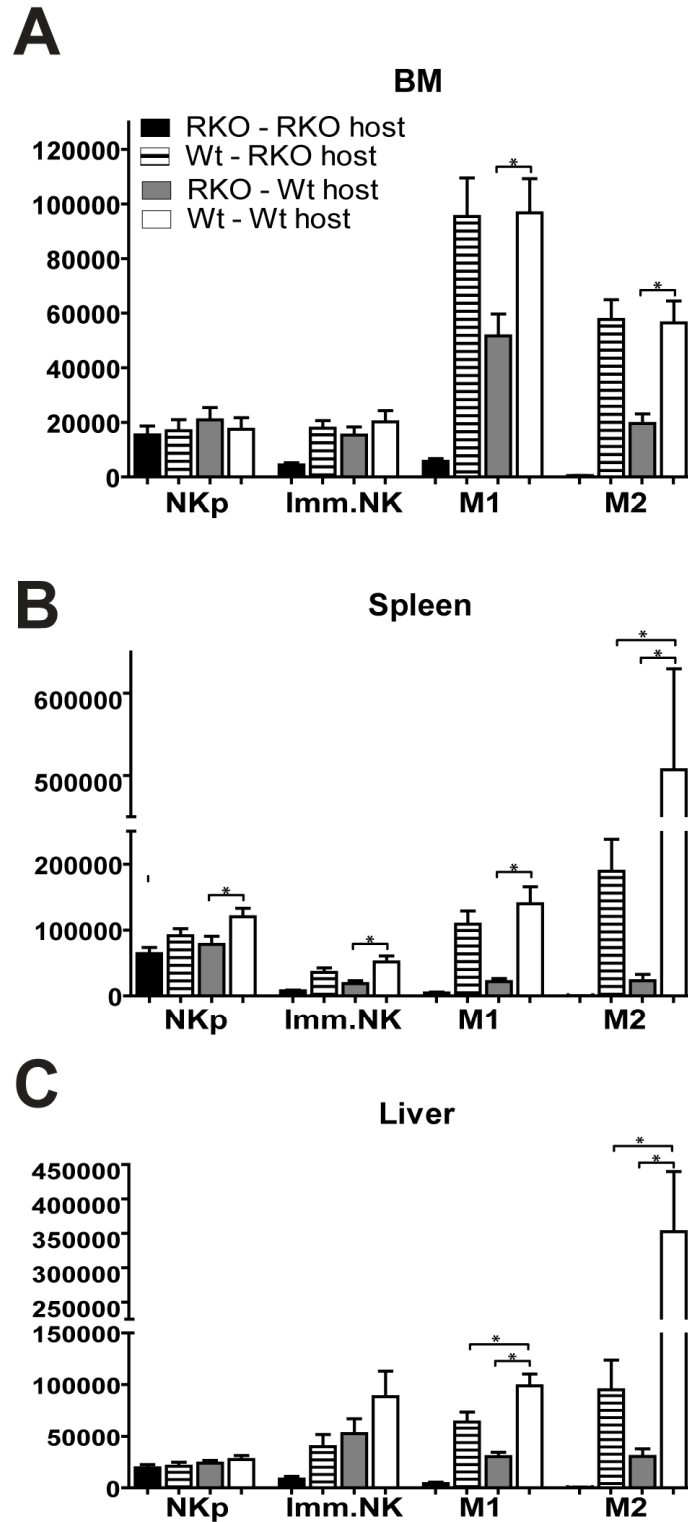
Because NK cell development involves the transition through multiple stages to become a mature NK cell <sup>47,48</sup>, the next step was to identify the stages of development regulated by each cellular compartment. This was accomplished by quantifying the following transitional subsets using differential expression of various cell surface markers. The phenotype of each stage were defined by the following cellular phenotype: NK precursors - CD122<sup>+</sup> NK1.1<sup>-</sup> DX5<sup>-</sup> CD3<sup>-</sup>; immature - CD122<sup>+</sup> NK1.1<sup>+</sup> DX5<sup>-</sup> CD3<sup>-</sup>; early mature [M1] - CD122<sup>+</sup> NK1.1<sup>+</sup> DX5<sup>+</sup> CD11b<sup>Low</sup> CD3<sup>-</sup>; and late mature [M2] - CD122<sup>+</sup> NK1.1<sup>+</sup> DX5<sup>+</sup> CD11b<sup>High</sup> CD3<sup>-</sup> NK cells (**FIGURE 9**). When IL-15R $\alpha$  expression was restricted to hematopoietic cells (WT BM  $\rightarrow$  IL-15R $\alpha$ <sup>-/-</sup> chimeras), the development of NK cells at all stages was completely recovered in the BM as compared with WT BM  $\rightarrow$  WT chimeras (**FIGURE 10**). In the liver and spleen, IL-15R $\alpha$ <sup>+</sup> hematopoietic cells significantly enhanced immature and M1 NK cells, but restoration of the M2 population was incomplete (**FIGURE 10**). When IL-15 trans-presentation is mediated by the non-hematopoietic compartment (RKO BM  $\rightarrow$  WT host) the immature NK cells could be substantially recovered in the BM, but the M1 and M2 NK cells were only minimally affected, whereas the non-hematopoietic cells had little role in the liver and spleen (**FIGURE 10**). Taken together, this shows that

**FIGURE 9. NK cell development in BM Chimeras.**



**FIGURE 9. NK cell development in BM Chimeras.** Representative flow cytometry data from the BM of the stages of NK cell development. NK precursors are characterized as CD122<sup>+</sup> NK1.1<sup>-</sup> cells; Immature are CD122<sup>+</sup> NK1.1<sup>+</sup> DX5<sup>-</sup>; Early mature (M1) display CD122<sup>+</sup> NK1.1<sup>+</sup> DX5<sup>+</sup> CD11b<sup>Low</sup> phenotype; and Late mature (M2) are a CD122<sup>+</sup> NK1.1<sup>+</sup> DX5<sup>+</sup> CD11b<sup>High</sup> population.

**FIGURE 10. Recovered developmental stages and tissue distribution of NK cells in IL-15R $\alpha$  BM chimeras.**





**FIGURE 10. Recovered developmental stages and tissue distribution of NK cells in IL-15R $\alpha$  BM chimeras.** Lymphocytes were isolated from the indicated tissues of IL-15R $\alpha$ <sup>-/-</sup> BM chimeras 8-12 weeks after irradiation and BM reconstitution. Numbers of NK cells at the various developmental stages in the respective tissues of IL-15R $\alpha$ <sup>-/-</sup> BM chimeras is shown and distinguished by various cell surface markers (average of 3 independent experiments, n= 8 mice per group) Error bars represent S.E., \* p  $\leq$  0.05.

hematopoietic cells are the dominate cell-type to trans-present IL-15 during NK cell development and they are crucial for the late stage of maturation especially in the BM, whereas the non-hematopoietic compartment appears to assist in the early stages of NK cell development and has no effect on late maturation.

To exclude the possibility that donor-derived BM non-hematopoietic cells were contributing to the effects we observed, the same scheme of generating BM chimeras was also performed using sorted lineage<sup>-</sup> CD45<sup>+</sup> cKit<sup>+</sup> Sca-1<sup>+</sup> stem cells. Likewise, this group of chimeras also demonstrated the importance of IL-15R $\alpha$  expression by hematopoietic cells in driving NK cell development (data not shown). Overall, the data demonstrates hematopoietic cells are the predominant cell-type to drive the differentiation of immature cells into mature NK cells via IL-15 trans-presentation. Additionally, these observations suggest IL-15R $\alpha$ <sup>+</sup> non-hematopoietic cells together with IL-15R $\alpha$ <sup>+</sup> hematopoietic cells are critical in the early events of NK cell differentiation; however, the former has a minimal role in late development.

#### 2.2.2 Exclusive expression of IL-15R $\alpha$ to CD11c<sup>+</sup> DCs partially restores NK cell numbers in BM and periphery.

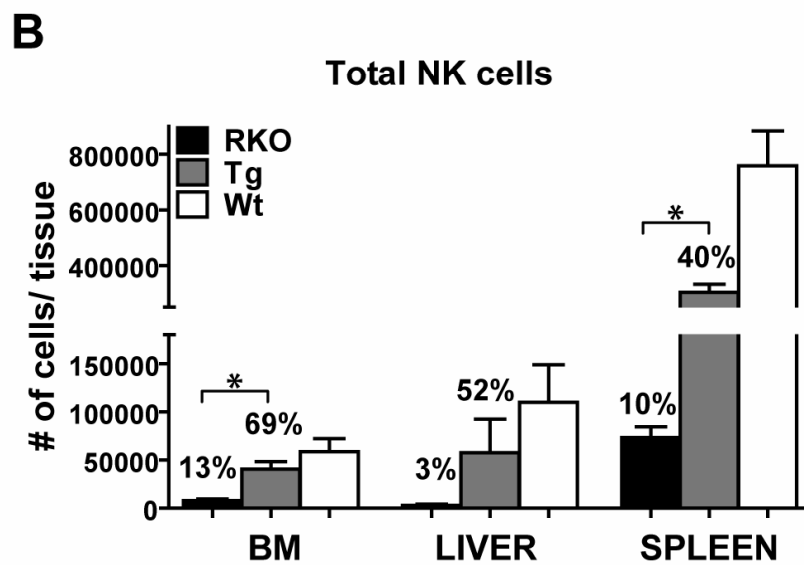
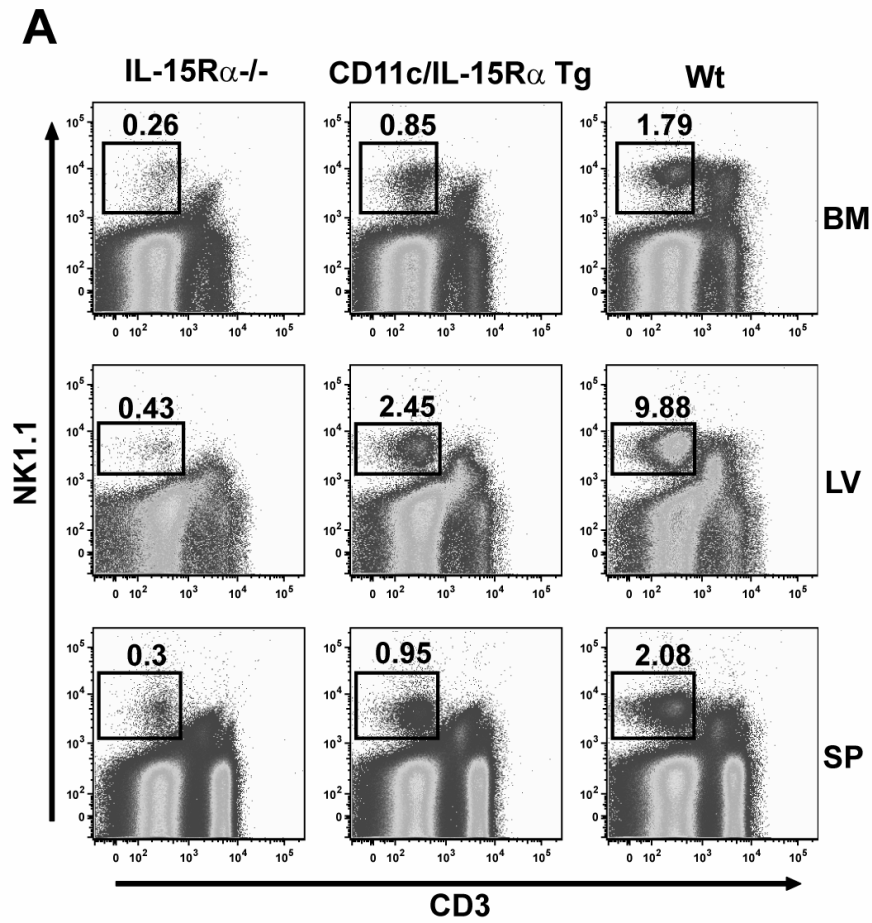
Since IL-15R $\alpha$ <sup>+</sup> hematopoietic cells emerge essential for NK cell development, we asked if DCs were the main cell-type trans-presenting IL-15 to NK cells. To determine how the effect of IL-15R $\alpha$  restricted to CD11c<sup>+</sup>

cells compares to the effect of IL-15R $\alpha$  expression by hematopoietic cells, recovery of the NK cells was examined in CD11c/IL-15R $\alpha$  Tg (Tg) mice bred on the IL-15R $\alpha$ <sup>-/-</sup> background<sup>35</sup>. This transgenic mouse restricts the expression of IL-15R $\alpha$  to CD11c<sup>High</sup> cells (**FIGURE 6**), thus can be utilized as a model where only DC can trans-present IL-15. As stated above, NK cells are present in the BM, liver and spleen, so each of these tissues were analyzed in the Tg mice and compared with IL-15R $\alpha$ <sup>-/-</sup> and WT (C57BL/6) mice for total NK1.1<sup>+</sup> CD3<sup>-</sup> cells. In each of these tissues, the frequency and total numbers of NK cells were significantly increased in the Tg mice compared with total numbers of NK cells in the IL-15R $\alpha$ <sup>-/-</sup> mice, with the recovery being most dramatic in the BM (**FIGURE 11**). These data reveal reconstitution of IL-15R $\alpha$  solely to the DC compartment contributes to the generation of total NK cell (NK1.1<sup>+</sup> CD3<sup>-</sup>) numbers.

### 2.2.3 IL-15 trans-presentation by DCs drives specific stages of NK cell development.

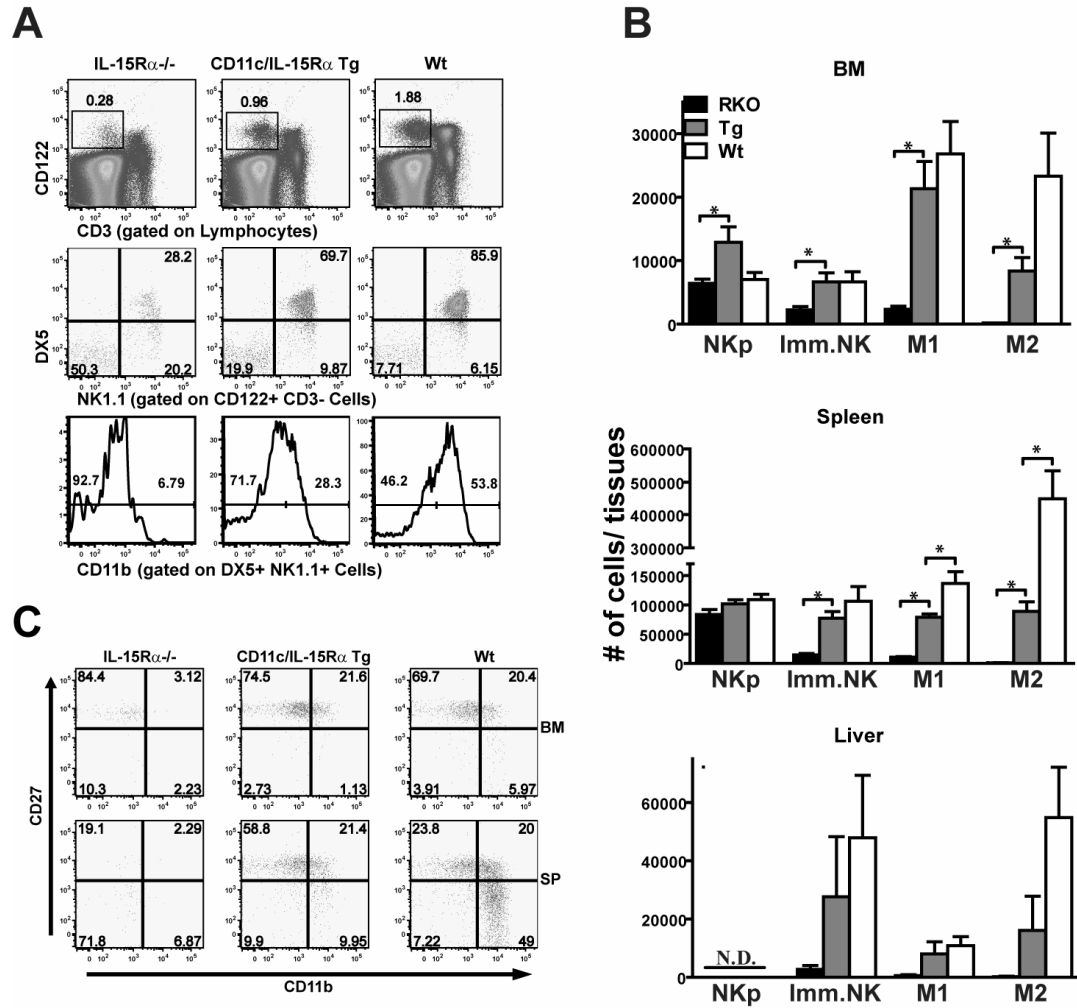
The next step was to identify the stages of NK cell development regulated by DCs. In all tissues examined, the complete absence of IL-15R $\alpha$  resulted in a dramatic decrease in the number of both of the mature (M1 and M2) NK cell subsets, a moderate decrease in immature NK cells but no deficiencies in NK precursors (**FIGURE 12**). Within the BM, IL-15 trans-presentation by DCs was very efficient in restoring the immature population and partially restored the early (M1) and late (M2) stages of NK cell development when compared

FIGURE 11. IL-15R $\alpha$ <sup>+</sup> DCs influence in the generation of NK cells.



**FIGURE 11. IL-15R $\alpha$ <sup>+</sup> DCs influence in the generation of NK cells.** *A*, NK1.1<sup>+</sup> CD3<sup>-</sup> cells recovered in the BM, liver and spleen of IL-15R $\alpha$ <sup>-/-</sup> (left column), CD11c/IL-15R $\alpha$  Tg (middle column) and B6 (right column) mice were analyzed by flow cytometry. Data is representative of three independent experiments. *B*, Absolute numbers of NK cells (NK1.1<sup>+</sup> CD3<sup>-</sup> cells) in the respective tissues of IL-15R $\alpha$ <sup>-/-</sup> (black bar), CD11c/IL-15R $\alpha$  Tg (gray bar) and B6 (white bar) mice were calculated (average of 3 independent experiments, n= 7 mice per group). Error bars represent S.E., \* p  $\leq$  0.05. Numbers above bars represent percent of Wt levels. **Copyright 2009. The American Association of Immunologist, Inc.**

**FIGURE 12. Recovered developmental stages and tissue distribution of NK cells by DC-mediated IL-15 trans-presentation.**



**FIGURE 12. Recovered developmental stages and tissue distribution of NK cells by DC-mediated IL-15 trans-presentation.** A-C, Stages of NK cell development were identified by flow cytometric analysis of BM, spleen, and liver isolated from each group of mice (IL-15R $\alpha$ <sup>-/-</sup>, CD11c/IL-15R $\alpha$  Tg, and Wt mice). Representative flow cytometry data from the BM is shown in panel A. B, absolute numbers of NK cells at the various developmental stages in the respective tissues (average of 3 independent experiments, n= 7 mice per group, Error bars represent S.E., \* p  $\leq$  0.05.) in the indicated groups. C, NK cell subsets distinguished by their cell surface expression of CD27 and CD11b in the BM (top row) and spleen (bottom row) from each group of mice. Flow cytometric plots are representative of three independent experiments (n=7 for each group of mice). **Copyright 2009. The American Association of Immunologist, Inc.**

with the IL-15R $\alpha$ <sup>-/-</sup> mice (**FIGURE 12b**). Nevertheless, this level of recovery in the BM is not equivalent to what was observed in WT BM → IL-15R $\alpha$ <sup>-/-</sup> chimeras and indicates that other non-CD11c<sup>-</sup> hematopoietic cells are essential in the transition into the M2 stage of development (**FIGURE 12b**). In the spleen, IL-15R $\alpha$ <sup>+</sup> DCs recovered immature NK cells and significantly enhanced both M1 and M2 populations (**FIGURE 12b**). Similar to the BM and spleen, the presence of IL-15R $\alpha$ <sup>+</sup> cells in the liver also enhanced the development of immature and mature NK cells but was most effective in recovering the M1 stage compared with WT mice (**FIGURE 12b**). These effects were not observed so much in the spleen and liver of IL-15R $\alpha$ <sup>-/-</sup> BM → WT chimeras, suggesting that CD11c<sup>+</sup> cells are the dominant hematopoietic cells trans-presenting IL-15 to NK cell in the periphery (**FIGURE 12b**)

Stages of mouse NK cell development and maturation have also been distinguished by the decreased expression of CD27 and the increased expression of CD11b with the earliest stage being the CD27<sup>+</sup>CD11b<sup>Low</sup>, which gives rise to a CD27<sup>+</sup>CD11b<sup>+</sup> population and eventually a terminally differentiated CD27<sup>Low</sup>CD11b<sup>+</sup> subset<sup>51</sup>. Therefore, we assessed the recovery of these three stages in the CD11c/IL-15R $\alpha$  Tg mice. In our analysis of CD27 and CD11b populations in the BM, similar proportions of CD27<sup>+</sup>/CD11b<sup>Low</sup> and the CD27<sup>+</sup>/CD11b<sup>+</sup> cells were observed in the CD11c/IL-15R $\alpha$  Tg and WT mice, whereas the majority of the NK cells in IL-



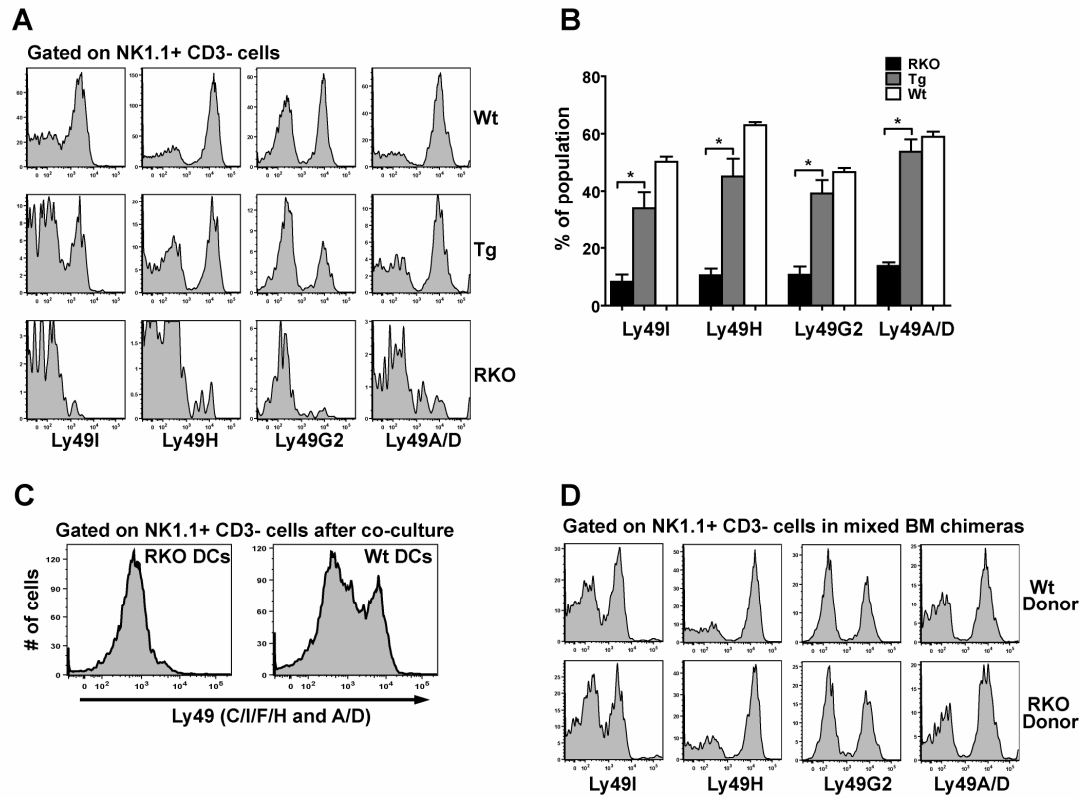
15R $\alpha$ <sup>-/-</sup> mice displays a CD27<sup>+</sup>CD11b<sup>Low</sup> phenotype (**FIGURE 12c**).

Conversely, whereas all three populations were prevalent in the spleen of WT mice, the CD27<sup>Low</sup>/CD11b<sup>+</sup> phenotype was deficient in the CD11c/IL-15R $\alpha$  Tg mice (**FIGURE 12c**). This analysis suggests that IL-15 trans-presentation by DC is insufficient for maturation of CD27<sup>Low</sup>/CD11b<sup>+</sup> NK cells, which preferentially reside outside the BM.

#### 2.2.4 IL-15R $\alpha$ <sup>+</sup> DC promotes the up-regulation of Ly49 receptors in developing NK cells.

Our findings so far demonstrate IL-15 trans-presentation by CD11c<sup>+</sup> DC subsets primarily act at the immature stage of NK cell development. During this stage, expression of activating and inhibitory receptors (CD94/NKG2 and Ly49), which are important for NK cell licensing, are induced<sup>44,45</sup>. Although there is evidence that both IL-15 and IL-15R $\alpha$  participate in the acquisition of Ly49 receptors<sup>31</sup>, the cell type providing IL-15 for the transition to a Ly49<sup>+</sup> population is unclear. Thus, the cell surface expression of various activating and inhibitory receptors by splenic NK cells was analyzed in IL-15R $\alpha$ <sup>-/-</sup>, CD11c/IL-15R $\alpha$  Tg, and WT mice. NK cells from CD11c/IL-15R $\alpha$  Tg and WT mice, but not IL-15R $\alpha$ <sup>-/-</sup> mice, expressed normal levels of the activating receptor Ly49H, as well as the inhibitory receptors Ly49G2 and Ly49 I (**FIGURE 13a and b**). Conversely, expression of CD94, NKG2 A/C/E, 2B4, and NKG2D was similar in the three groups of mice indicating that their

**FIGURE 13. DCs drive Ly49 expression of immature NK cells.**



**FIGURE 13. DCs drive Ly49 expression of immature NK cells.** *A and B*, Expression of Ly49 activating and inhibitory receptors on splenic NK cells as detected by flow cytometry in WT (top row), CD11c/IL-15R $\alpha$  Tg (middle row) and IL-15R $\alpha$ <sup>-/-</sup> (bottom row) mice. *A*, histograms showing representative data from two independent experiments. *B*, graph depicts the average percent of the population that is positive for the indicated Ly49 molecule (n=4 per group). Error bar represent S.E., \* p ≤ 0.05.). *C*, *in vitro* acquisition of the Ly49 repertoire by BMDC. Ly49<sup>-</sup> NK cells were sorted from spleens of WT mice and cocultured with DCs generated from BM of either IL-15R $\alpha$ <sup>-/-</sup> (left plot) or WT (right plot) mice with GM-CSF. Plots show expression of Ly49 on NK1.1<sup>+</sup> CD3<sup>-</sup> cells at the end of coculturing. Data are representative of two independent experiments. *D*, cell surface expression of Ly49 activating and inhibitory receptors by NK cells derived from Wt (top row) and IL-15R $\alpha$ <sup>-/-</sup> (bottom row) BM donor cells. BM cells from Wt (CD45.1<sup>+</sup>/CD45.2<sup>+</sup>) and IL-15R $\alpha$ <sup>-/-</sup> (CD45.2<sup>+</sup>) were injected in equal proportions into irradiated CD45.1<sup>+</sup> Wt hosts. After reconstitution of hematopoietic cells (~8-10 weeks later), the respective donor-derived NK1.1<sup>+</sup> CD3<sup>-</sup> cells in the spleen were identified based on CD45 isoform expression. Plots are representative of three experiments (n=5 mice/group). **Copyright 2009. The American Association of Immunologist, Inc.**

acquisition is independent of IL-15 (data not shown). This data suggest that IL-15 trans-presentation by CD11c<sup>+</sup> DCs up-regulates the expression of the Ly49 repertoire on NK cells in vivo.

To directly demonstrate that DCs drive the differentiation of Ly49<sup>-</sup> NK cells into Ly49<sup>+</sup> NK cells, we determined whether IL-15 trans-presentation by purified DCs could drive this differentiation process in vitro. Therefore, Ly49<sup>-</sup> NK cells were isolated from WT splenocytes by flow cytometric sorting and then cocultured with BM-derived DCs from either IL-15R $\alpha$ <sup>-/-</sup> or WT mice. After a 5-day incubation period, NK cells cocultured in the presence of IL-15R $\alpha$ <sup>+</sup> WT DCs expressed the various Ly49 markers whereas NK cells cultured with IL-15R $\alpha$ <sup>-/-</sup> DCs did not (**FIGURE 13c**), supporting our in vivo findings that DC-mediated IL-15 trans-presentation drives a specific differentiation event during NK cell development.

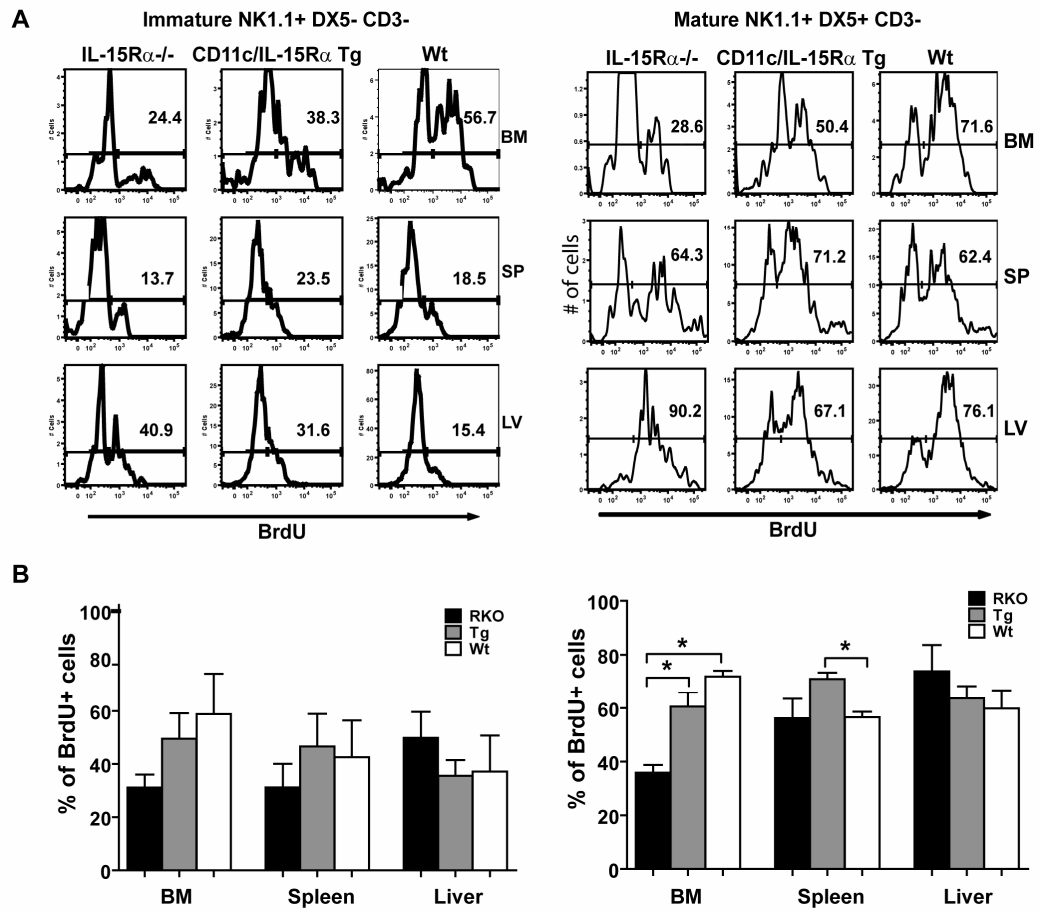
To exclude the possibility that IL-15R $\alpha$  expression by NK cells is required for Ly49 expression as suggested by previous studies<sup>67</sup>, mixed BM chimeras were generated by injecting equal amounts of IL-15R $\alpha$ <sup>-/-</sup> BM (CD45.1<sup>+</sup>/CD45.2<sup>+</sup>) and WT BM (CD45.2<sup>+</sup>) into lethally irradiated congenic WT mice (CD45.1<sup>+</sup>). After reconstitution of the hematopoietic compartment, the various Ly49 receptors (I, H, G2, and A/D) were expressed at similar levels by NK cells derived from either IL-15R $\alpha$ <sup>-/-</sup> and WT cells coexisting in the same mice (**FIGURE 13d**). This finding demonstrates that IL-15R $\alpha$  expression by NK cells is not necessary for Ly49 up-regulation. Altogether,

these findings reveal IL-15 trans-presentation is critical in the acquisition of the activating and inhibitory Ly49 receptors, and DCs are capable of driving this specific differentiation event required for functional maturation.

#### 2.2.5 Proliferation of NK cells is restored by DC-mediated IL-15 trans-presentation.

During NK cell development, expansion of successfully differentiated NK cells is an important component of NK cell development. Since IL-15 is known to induce proliferation<sup>68,71</sup>, we determined whether IL-15R $\alpha$ <sup>+</sup> DCs affects cell expansion occurring during NK cell development. To examine this role, the amount of BrdU incorporation in the immature (NK1.1<sup>+</sup> DX5<sup>-</sup> CD3<sup>-</sup>) and mature (NK1.1<sup>+</sup> DX5<sup>+</sup> CD3<sup>-</sup>) NK cells was compared among the three groups of mice. In the BM, BrdU incorporation by NK cells in IL-15R $\alpha$ <sup>-/-</sup> was dramatically low in both immature and mature populations in comparison to WT mice (**FIGURE 14a and b**). In Tg mice, IL-15R $\alpha$ <sup>+</sup> DCs enhanced the incorporation of BrdU by both immature and mature NK cells found in the BM (**FIGURE 14a and b**). In contrast, the absence of IL-15R $\alpha$  or the presence of IL-15R $\alpha$ <sup>+</sup> DCs had no affect on peripheral NK cell proliferation (**FIGURE 14a and b**). Collectively, the above data show the expansion of mature NK cells occurring in the BM is dependent on IL-15 trans-presentation and partially driven by CD11c<sup>+</sup> DCs.

**FIGURE 14. DCs assist in the expansion of NK cells in the BM.**



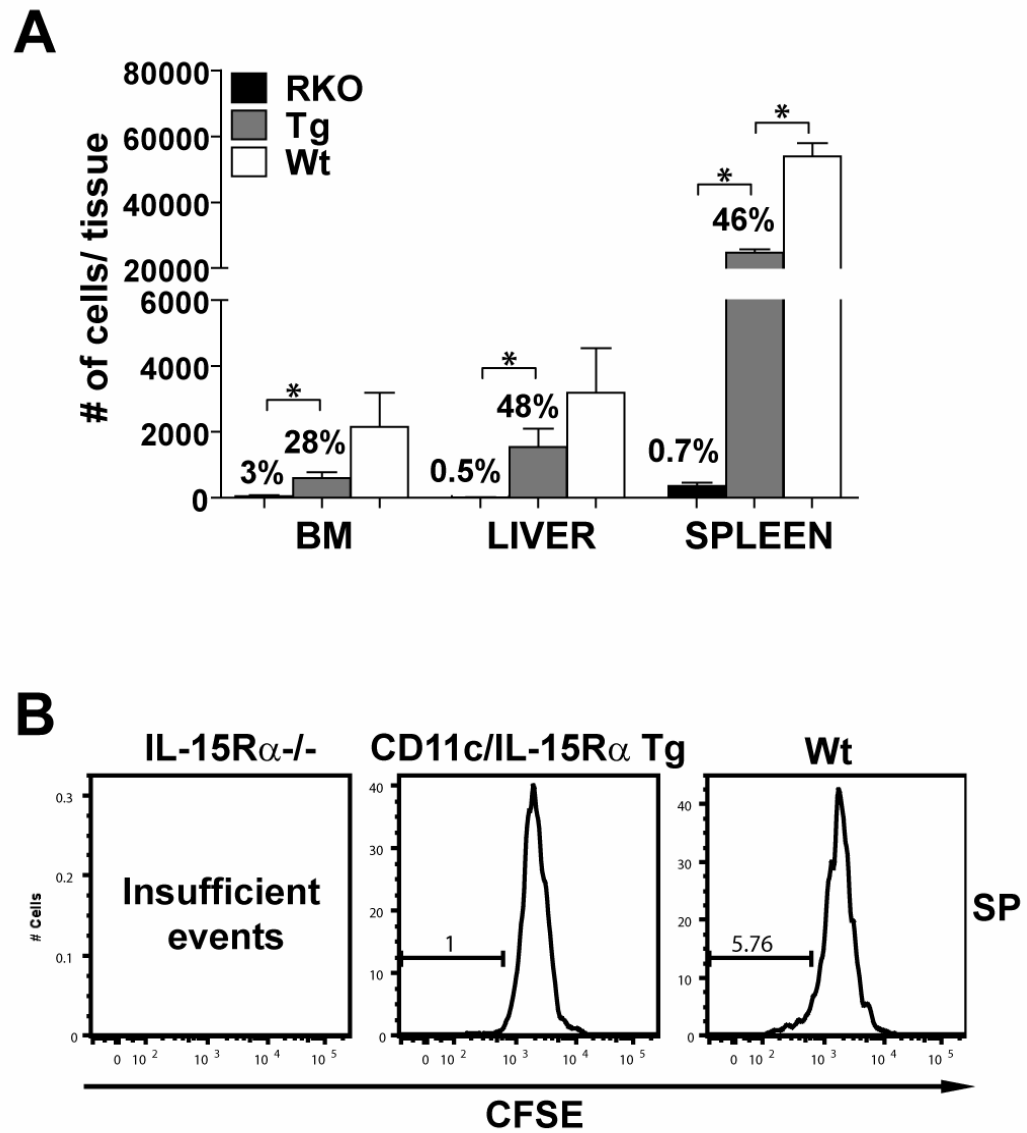
**FIGURE 14. DCs assist in the expansion of NK cells in the BM.** *A*, Representative histograms showing the *in vivo* BrdU incorporation (3 weeks of BrdU [0.8mg/ml] in drinking water) as detected by flow cytometry in (A) immature (NK1.1<sup>+</sup> DX5<sup>-</sup> CD3<sup>-</sup>) and mature NK cells (NK1.1<sup>+</sup> DX5<sup>+</sup> CD3<sup>-</sup>) found in the BM (top row), spleen (middle row), and liver (bottom row) in the three groups of mice. *B*, Graph depicts the average BrdU incorporation in immature and mature NK cells from the individual groups in two independent experiments (n=4 mice/group). **Copyright 2009. The American Association of Immunologist, Inc.**

### 2.2.6 Survival of mature NK cells is mediated by DCs trans-presenting IL-15.

After development is complete, maintenance of mature NK cells is predominately mediated by survival of NK cells, rather than by homeostatic proliferation, and is highly dependent on IL-15 trans-presentation<sup>32,68,71,111</sup>. Lymphopenia-induced proliferation of NK cells also uses IL-15 trans-presentation and has recently been shown to be mediated by DCs<sup>123</sup>. Whether DCs also maintain the survival of mature NK cells via IL-15 trans-presentation under steady-state conditions has not been demonstrated. To examine the role of IL-15 trans-presentation by DCs in NK cell homeostasis, splenic NK cells from WT mice (CD45.1<sup>+</sup>) were labeled with CFSE and transferred into IL-15R $\alpha$ <sup>-/-</sup>, CD11c/IL-15R $\alpha$  Tg, and WT mice (all CD45.2<sup>+</sup>). After 3 wk, the BM, liver, and spleen were extracted and analyzed for the presence of donor NK cells (**FIGURE 15a**). In all tissues examined, the number of donor NK cells was severely decreased (between 0.5 and 3% of WT levels) in IL-15R $\alpha$ <sup>-/-</sup> hosts compared with that found in WT mice highlighting the crucial role of IL-15R $\alpha$  in NK cell survival (**FIGURE 15a**). In CD11c/IL-15R $\alpha$  Tg mice, the number of donor NK cells was recovered to 46 and 48% of WT levels in the liver and spleen, respectively. Examination of cell division via CFSE dilution indicated homeostatic proliferation did not occur during this time (**FIGURE 15b**), as reported previously<sup>32,70</sup>. Since recovery of donor NK cells was not as prevalent in the BM of CD11c/IL-15R $\alpha$  Tg mice, it suggests that either mature NK cells do not migrate to the BM as efficiently as to the spleen or liver or that either CD11c<sup>+</sup> cells in the BM do not



**FIGURE 15. DCs support the maintenance of peripheral NK cells.**



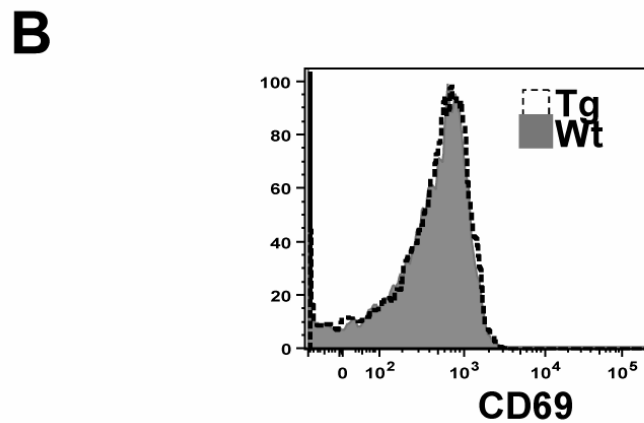
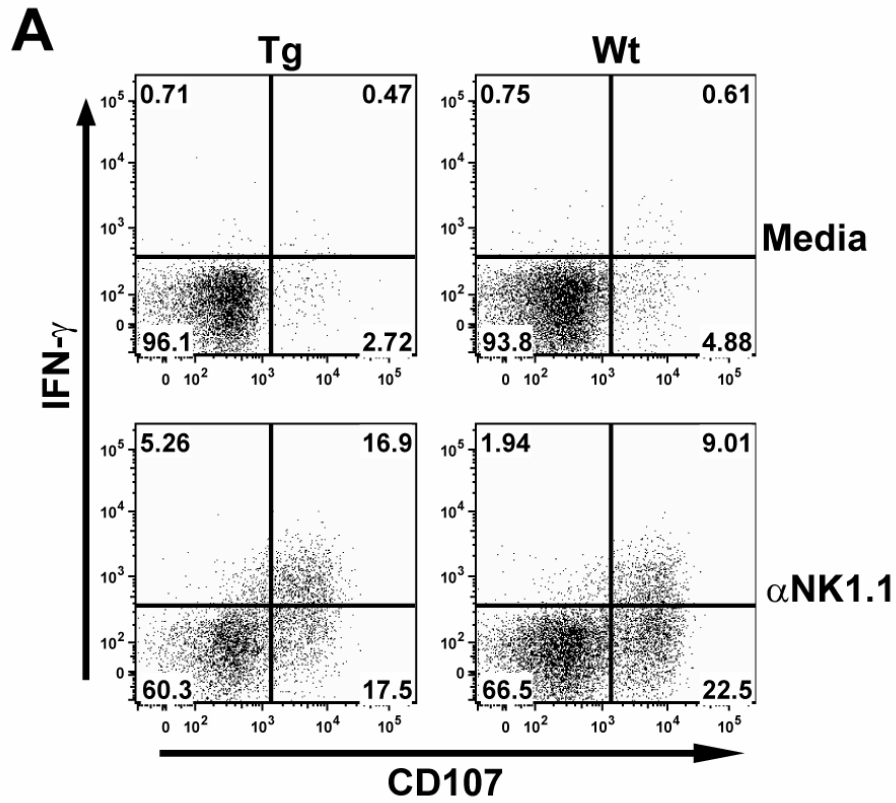
**FIGURE 15. DCs support the maintenance of peripheral NK cells.** *A*, Graph shows the average numbers of donor NK cells (gated on CD45.1<sup>+</sup> NK1.1<sup>+</sup> DX5<sup>+</sup> CD11b<sup>+</sup> CD3<sup>-</sup> cells) recovered in the BM, spleen, and liver three weeks after the adoptive transfer into congenic CD45.2<sup>+</sup> IL-15R $\alpha$ <sup>-/-</sup> (black bar), CD11c/IL-15R $\alpha$  Tg (gray bar) and WT (white bar) mice. Error bars represent S.E. and the numbers above bars represent percent of Wt levels, \*  $p \leq 0.05$ . *B*, Dilution of CFSE by donor cells found in the spleen of the three hosts. Plots are representative of two individual experiments (n=4 for each group). **Copyright 2009. The American Association of Immunologist, Inc.**

provide survival signals to mature NK cells. Altogether, the data demonstrate that DCs indeed participate in mediating survival of mature NK cells by trans-presenting IL-15.

#### 2.2.7 NK cells developing in CD11c/IL-15R $\alpha$ Tg mice are functional and do not show signs of aberrant activation.

The highlight of NK cell development is the generation of NK cells that can mount an early immune response regulated by cytokine activation or down-regulation of self MHC class Ia<sup>38</sup>. NK cells recovered by IL-15R $\alpha$ <sup>+</sup> DCs display a phenotype that is associated with functional maturation<sup>47</sup>. Since recent reports<sup>73,74</sup> demonstrate that DCs can activate NK cells via IL-15 trans-presentation, it is possible that Tg expression of IL-15R $\alpha$  to DCs could lead to aberrant NK cell activation. To examine the activation and functional status, NK cells were sorted from CD11c/IL-15R $\alpha$  Tg and WT mice and stimulated with plate-bound NK1.1 Ab or media alone<sup>44</sup>. After a 5-h incubation period, the production of IFN- $\gamma$  and cytolytic potential as determined by CD107 $\alpha$  cell surface mobilization was analyzed. Similar to WT NK cells, NK cells from the CD11c/IL-15R $\alpha$  Tg mice produced IFN- $\gamma$  and expressed CD107 $\alpha$  only in response to NK1.1 stimulation (**FIGURE 16a**). In addition, the expression level of the activation-enhanced cell surface marker, CD69, by unmanipulated NK cells from the CD11c/IL-15R $\alpha$  Tg mice was similar to WT NK cells (**FIGURE 16b**). Taken together, our data suggest DC-

**FIGURE 16. NK cells derived by DC-mediated IL-15 trans-presentation are functional but not activated under steady-state conditions.**



**FIGURE 16. NK cells derived by DC-mediated IL-15 trans-presentation are functional but not activated under steady-state conditions. A,**

Effector function of NK cells. NK cells sorted from spleens of either WT or CD11c/IL-15R $\alpha$  Tg mice were incubated for five hours in the absence (top row) or presence (bottom row) of plate-bound anti-NK1.1 antibody.

Intracellular IFN- $\gamma$  production and CD107 cell surface mobilization were measured via flow cytometry as indicators of effector function. Plot is

representative of two independent experiments. *B*, *Ex vivo* comparison of the cell surface expression of the activation-induced marker, CD69, by splenic

NK cells from Wt or CD11c/IL-15R $\alpha$  Tg mice. ***Copyright 2009. The American Association of Immunologist, Inc.***

mediated IL-15 trans-presentation during steady-state conditions does not activate NK cells but rather generates functionally mature NK cells.

## 2.3 Discussion

Interactions occurring between DC and NK cells have typically been described in the context of an immune response<sup>124</sup>. For example, it has been reported that DC-mediated IL-15 trans-presentation activates NK cells both in vitro and in vivo<sup>73,74</sup>. But whether DCs also participate in NK cell ontogeny by providing IL-15 has not been described. In this chapter, I provide evidence that DC indeed participate in NK cell development as well as in the homeostatic maintenance of NK cells via IL-15 trans-presentation. Furthermore, I identify specific stages of NK cell development where IL-15 trans-presentation by CD11c<sup>+</sup> and CD11c<sup>-</sup> hematopoietic cells preferentially occurs highlighting an important role for cell-specific IL-15 trans-presentation in NK cell functional maturation.

BM stromal cells have long been regarded as the important cell-type controlling NK cell development<sup>64-66</sup>. Therefore, it may be unexpected that DCs also mediate some of the functions previously attributed to BM stromal cells. Nonetheless, the previous studies examining BM stromal cells did not exclude the possibility that other cell types have overlapping functions. Confusion can also arise as a result of ambiguous and generic terminology used to define stromal cells. Although "stromal" is generally used to describe structural support cells, the more accepted definition regarding BM stromal cells refers to radiation resistant cells, which are believed to be non-hematopoietic. In contrast, others have described BM stromal cells as

fibroblast-like cells that can be isolated from the BM but can reconstitute hematopoietic cells <sup>125</sup>. More importantly, the conclusions made in this present study are based on the definition of BM stromal cells that was used in prior studies i.e., activity attributed to radiation-resistant cells in BM chimera models <sup>64-66</sup>. Overall, the present study adds to these previous findings showing other cell types, which are CD11c<sup>+</sup> can also provide IL-15 to NK cells during early events of development.

I can only speculate as to why NK cells use IL-15 by both non-hematopoietic and hematopoietic cells. The BM is an abundant site for hematopoiesis. Since non-hematopoietic BM stromal cells are also involved in the development of other cell types such as B cells <sup>126</sup>, it is possible that there is competition to receive resources from this cell type. Therefore, it would be advantageous for NK cells if they weren't completely dependent on stromal cells for their IL-15. Second, IL-15 is up-regulated by TLR ligands <sup>127</sup>, which are a component of the adaptive response to pathogens. Since DCs are very efficient in responding to TLR stimulation, DCs may have evolved as the predominant cell-type expressing IL-15. With time, the necessity for IL-15 to be stimulated by TLR was lost by DCs, leaving this cell type as a promoter of NK cell development.

In this present study, I specifically demonstrate IL-15R $\alpha$ <sup>+</sup> DCs are efficient in driving the differentiation of immature and DX5<sup>+</sup> NK cells (M1) and inducing acquisition of Ly49 expression while having a minor role in the transition of



late stages of NK cell development (i.e., up-regulating CD11b and down regulating CD27). This role of DCs at specific stages of NK cell development suggests that distinct niches of NK cell development likely exist in the BM. Because conventional DCs are present in the BM at a low frequency and CD11c<sup>+</sup> cells are a heterogeneous population containing CD11c<sup>+</sup> plasmacytoid DCs and DC precursors<sup>128,129</sup>, the precise phenotype of the cells mediating these activities is not clear. In our hands, detection of cell surface IL-15 and IL-15R $\alpha$  by CD11c<sup>+</sup> BM cells is found by putative DC precursors but not plasmacytoid DCs. Thus, a cell type contained within the putative DC precursor population may be a DC subset mediating IL-15 trans-presentation. Despite the effectiveness of DCs in providing IL-15 during the early stages of NK cell development, trans-presentation of IL-15 by CD11c<sup>+</sup> cells alone was insufficient in mediating all the IL-15 events attributed to IL-15R $\alpha$ <sup>+</sup> hematopoietic cells, especially in the late stages of NK cell development. This finding highlights an unrecognized role for other CD11c<sup>-</sup> hematopoietic cells in NK cell development that may not have been previously identified; this may be because the late stages of NK cell development are not crucial for functional maturation but rather for mediating NK cell expansion. Identifying these other IL-15 trans-presenting hematopoietic cells involved in NK cell development may provide future insight on how best to improve NK cell expansion.

In the peripheral tissues, the restricted expression of IL-15R $\alpha$  to CD11c<sup>+</sup> cells had less of an impact on recovery of normal NK cell numbers than in the BM.

Because the effects of limiting IL-15R $\alpha$  expression to CD11c<sup>+</sup> cells was similar to that when restricted to all hematopoietic cells, we speculate that conventional DCs are the predominate hematopoietic cells mediating IL-15 trans-presentation outside of the BM. On the basis of my data, the function of IL-15 trans-presentation in these peripheral tissues is more geared toward the homeostatic maintenance of NK cells. With the presence of precursors and developmental intermediates located in the peripheral tissues, it is possible that NK cell developmental niches can occur outside of the BM, but whether some residual differentiation occurs in these sites is still uncertain.

The functional attributes of NK cells are greatly dependent on acquiring various activating and inhibitory receptors<sup>44</sup>. The expression of these receptors, specifically the various Ly49 receptors, has been shown to be dependent on NK cells expressing IL-15R $\alpha$ <sup>31</sup>. In contrast, analysis from our Tg mice and the BM chimera models demonstrate that Ly49 expression is independent of IL-15R $\alpha$  expression by NK cells. Past research has suggested that the addition of only IL-15 to NKp cultures drove the differentiation of NK cells; however, this differentiated population was negative for the Ly49 repertoire<sup>130</sup>. The up-regulation of the Ly49 repertoire appeared by NK cells only in the presence of stromal cells and IL-15<sup>131,132</sup>. These observations suggested that a cell-cell interaction was needed to acquire the Ly49 receptors, and IL-15 alone was not enough. More recently, Huntington et al.<sup>133</sup> revealed that although IL-15 provides survival signals to NK cells, the up-regulation of killer Ig-related receptors by NK cells was

independent of such survival signals and dependent on IL-15 trans-presentation. Thus, we add new insight into the acquisition of the Ly49 repertoire by showing that DC-mediated IL-15 trans-presentation can drive this event previously shown to be mediated by BM stromal cells <sup>131,132</sup>; it also provides further support for the notion that IL-15 is essential in up-regulating the Ly49 repertoire. More importantly, since acquiring the various Ly49 inhibitory receptors is crucial in NK cell education, our findings demonstrate that IL-15 trans-presentation by DCs participates in a crucial first step for this event. In addition to the early requirement for Ly49 expression, DCs may directly mediate NK cell education as DCs express the various ligands that interact with NK cell receptors <sup>20</sup>. Interestingly, since it has been reported that IL-15 up-regulates MHC class I Ag-processing machinery in human DCs <sup>134</sup>, IL-15 may also regulate NK cell education by acting on the DCs to promote the expression of ligands for Ly49.

After NK cell education, expansion of maturing NK cells is an important late event in the developmental process. In my findings using transfers of mature NK cells, NK cell proliferation was not observed, which is consistent with previous studies showing maintenance of mature NK cells is mediated by survival <sup>32,70</sup>. However, since mature NK cells in the periphery were BrdU<sup>+</sup>, I reason that cell division occurred before exiting the BM. Since I observed that the numbers of late-stage mature NK cells and proliferation were deficient in the CD11c/IL-15R $\alpha$  Tg mice, one could have suspected that licensing and/or functional maturation is incomplete. In addition, licensed NK cells are also

believed to possess enhanced proliferation potential over unlicensed NK cells<sup>44</sup>. However, since I demonstrate that NK cells from CD11c/IL-15R $\alpha$  Tg mice have normal Ly49 expression and functional attributes, I believe these NK cells are functionally mature but have not received an adequate expansion signal. Overall, the minor loss in proliferation appears to be independent of NK cell licensing and possibly dependent on other IL-15R $\alpha$ <sup>+</sup> cell-types.<sup>135</sup>

After the publication of my findings<sup>135</sup>, Mortier et al<sup>122</sup> reported the phenotype observed in IL-15R $\alpha$  flox mice interbred to CD11c-Cre Tg (deletion of IL-15R $\alpha$  in DCs) or LysM-Cre Tg (deletion of IL-15R $\alpha$  in macrophages) mice. In both models, peripheral NK cells were reduced in numbers but not affect was observed for BM NK cells. Specifically, a reduction in the total number of mature CD27<sup>-</sup> CD11b<sup>+</sup> splenic NK cells was observed and attributed to a loss in CD27<sup>+</sup> CD11b<sup>+</sup> NK cell proliferation. Consequently, it was concluded that DCs (and macrophages) were crucial for the generation of mature peripheral NK cells. Similarly, my data revealed this mature CD27<sup>-</sup> CD11b<sup>+</sup> NK population was also reduced in the periphery and BM but the models used in my study, had either DCs as the only cell-type expressing IL-15R $\alpha$  or all hematopoietic cells expressing IL-15R $\alpha$ <sup>135</sup>. Thus, it is unlikely that only DCs and macrophages are required for this late stage of NK cell development as stated by Mortier and colleagues<sup>122</sup>. Furthermore, the CD11c promoter has been shown to spill over into non-DCs including monocytes, macrophages, neutrophils, NK, B and T cells.

Regarding NK cell homeostasis, Mortier et al <sup>122</sup> revealed DCs and macrophages had no role in the homeostatic maintenance of NK cells; however, I revealed DCs play a significant role in the maintenance of peripheral NK cells. These different results could be due to the time points being assessed: Mortier et al <sup>122</sup> analyzed the recovery of NK cells at 4 hour, 2 and 7 days and I analyzed the recovery of NK cells 21 days post-transfer <sup>135</sup>. It is plausible that donor NK cells transferred into CD11c-Cre/IL-15R $\alpha$  floxed mice would decline in number if mice were analyzed after a longer time period. Overall, both reports reveal: i) DCs (or macrophages) are capable of trans-presenting IL-15 during NK cell development but are not the single cell-type crucial for the generation of mature NK cells; and ii) the development and homeostasis of NK cells is a complex and continuous event that is regulated by IL-15 trans-presentation by cells of both hematopoietic and non-hematopoietic origin.

In conclusion, I have identified the cell-types regulating the development and homeostasis of NK cells via IL-15 trans-presentation in the various tissue microenvironments occupied by NK cells.

## **CHAPTER 3**

### **The Role of IL-15 Trans-Presentation During the Development and Homeostasis of Invariant Natural Killer T Cells.**

#### **3.1 Introduction**

The compelling features of iNKT cells, differential cytokine production and subsequent immune regulation, make these innate-like cells influential in various immunological responses. The involvement of iNKT cells in these responses can be advantageous or harmful to the host. The recent finding that various glycolipids can modify the cytokine production of iNKT cells enhances the potential of iNKT cells in the clinic. Nevertheless, a full comprehension of how the development and homeostasis of iNKT cells occur is lacking.

Since iNKT cells are very similar to NK cells with regards to IL-15-dependency it is likely IL-15 regulates other iNKT developmental events like functional maturation. Furthermore, since tissue-specific iNKT cells exist with varying functional attributes it is vital to understand the cellular cues the microenvironment provides during the development and homeostasis of iNKT cells. Thus, identifying the source of IL-15 in each iNKT cell microenvironment will prove fundamental in understanding the true nature of IL-15 in iNKT cell biology.

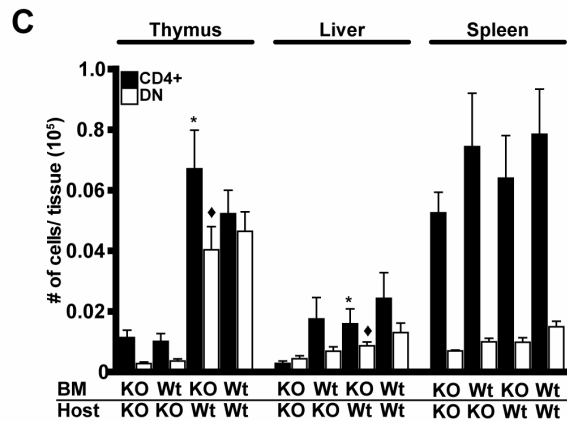
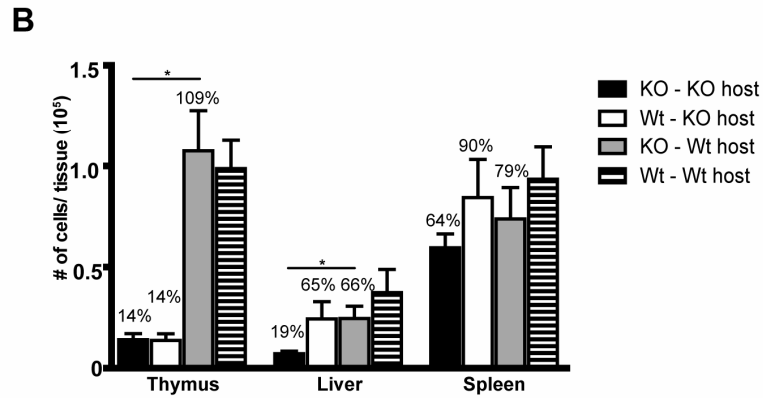
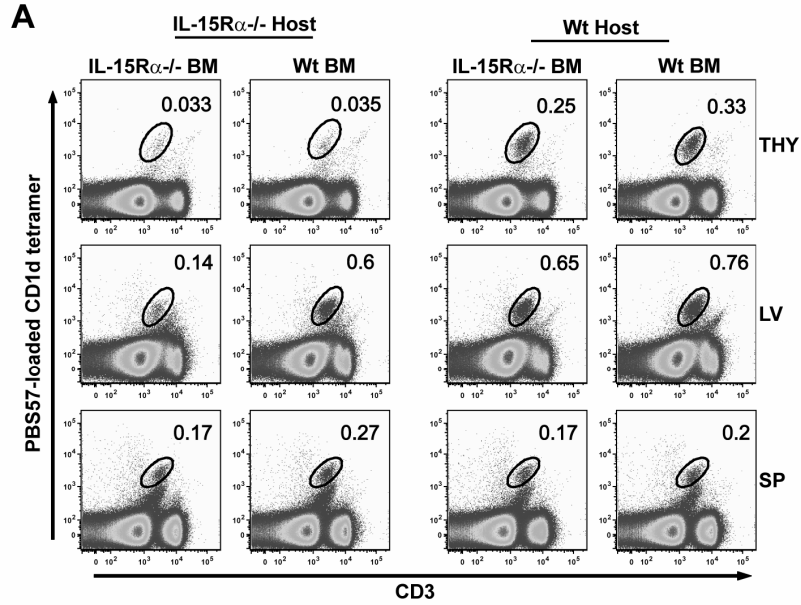
Presently, the exact role of IL-15 during the development and homeostasis of iNKT cells is unclear as is the source of IL-15. Therefore, I have identified the cell-types driving the differentiation, proliferation, functional maturation and homeostasis of iNKT cells via IL-15 trans-presentation in the various iNKT cell tissue microenvironments.

### 3.2 Results

#### “ 3.2.1 Radiation-resistant cells are crucial in providing IL-15 during intrathymic iNKT cell development.”

Presently it is unclear which cells require IL-15R $\alpha$  expression for iNKT cell development. Therefore, BM chimeras were generated to assess the role of IL-15 trans-presentation by hematopoietic or non-hematopoietic cells in restoring thymic and peripheral iNKT cells. Accordingly, iNKT cells were detected in the thymus, liver, and spleen in the 4 groups of BM chimera mice using CD1d-tetramers (**FIGURE 17**). IL-15R $\alpha$  expression by non-hematopoietic cells (IL-15R $\alpha$ <sup>-/-</sup>BM→WT host) recovered the frequency and total numbers of iNKT cells in the thymus similar to that found in WT chimeras (WT BM→WT) (**FIGURE 17b**). Conversely, the frequency and absolute numbers of thymic iNKT cells were still very much deficient when IL-15R $\alpha$  expression was expressed solely by hematopoietic cells (WT BM→IL-15R $\alpha$ <sup>-/-</sup>) to a level that was similar to IL-15R $\alpha$ <sup>-/-</sup> chimeras (IL-15R $\alpha$ <sup>-/-</sup>BM→IL-15R $\alpha$ <sup>-/-</sup>) (**FIGURE 17b**). Among peripheral iNKT cells in the liver, both IL-15R $\alpha$ <sup>+</sup> hematopoietic or non-hematopoietic cells significantly increased the

**FIGURE 17. Tissue-specific recovery of iNKT cells in IL-15R $\alpha$  BM chimeras**





**FIGURE 17. Tissue-specific recovery of iNKT cells in IL-15R $\alpha$  BM**

**chimeras. A-C,** Tissue-resident iNKT cells or iNKT subsets were identified by flow cytometric analysis in the thymus, spleen, and liver of IL-15R $\alpha$ <sup>-/-</sup> BM chimeras 8-10 weeks after irradiation and BM reconstitution by flow cytometric analysis. **A.** representative flow cytometric plots of CD1d-tetramer<sup>+</sup>CD3<sup>+</sup> cells from the indicated tissues. **B,** absolute numbers of CD1d-tetramer<sup>+</sup> cells in the respective tissues from the indicated groups. Error bars represent S.E.M. and the numbers above bars represent percent of WT levels \*  $p \leq 0.05$ . Data were average from 3 independent experiments, n= 9 mice per group for the thymus; n=8 for the liver and spleen. Statistical significance was determined by unpaired Student t test. **"This research was originally published in *Blood* Online. Eliseo F. Castillo, Luis F. Acero, Spencer W. Stonier, Dapeng Zhou, and Kimberly S. Schluns Thymic and peripheral microenvironments differentially mediate development and maturation of iNKT cells by IL-15 transpresentation *Blood* First Edition Paper, prepublished online June 25, 2010;DOI 10.1182/blood-2010-03-277103."**

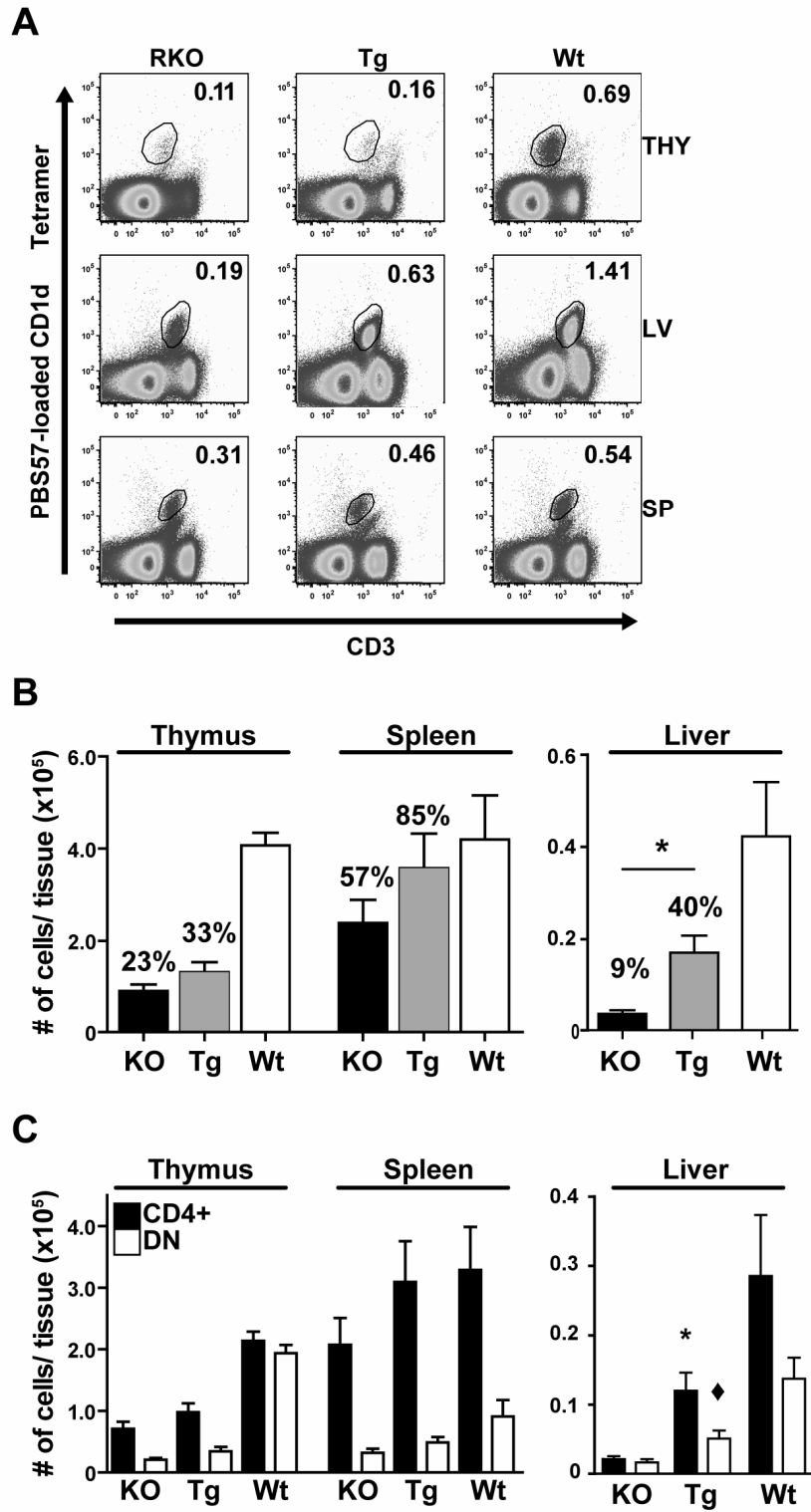
levels of iNKT cells (~65% of control) compared to that in the complete absence of IL-15R $\alpha$  (~19% of control) ( $p < 0.05$ ) (**FIGURE 17b**). In contrast to hepatic iNKT cells, splenic iNKT cells had minimal requirements for IL-15R $\alpha$  as the number of iNKT cells in the complete absence of IL-15R $\alpha$  was ~64% of control levels. While the numbers of splenic iNKT cells were increased by both IL-15R $\alpha$ <sup>+</sup> hematopoietic or non-hematopoietic cells, these differences were not significant. Surprisingly, IL-15R $\alpha$ <sup>+</sup> hematopoietic cells increased the total number of NKT cells in the liver and spleen albeit the reduction of iNKT cell numbers in the thymus (**FIGURE 17b**). In addition, even though normal numbers of iNKT cells were produced in the thymus by non-hematopoietic cells, expression of IL-15R $\alpha$  in the periphery by both hematopoietic and non-hematopoietic cells was required to maintain normal levels of iNKT cells (**FIGURE 17b**). Regarding CD4<sup>+</sup> and DN iNKT subsets, both subsets were equally dependent on IL-15R $\alpha$  expression despite cell-restricted expression (data not shown). Collectively, these findings suggest thymic iNKT cell development utilizes the non-hematopoietic cell compartment for IL-15 trans-presentation while peripheral iNKT cells receive IL-15 signals from both non-hematopoietic and hematopoietic cells. Remarkably, peripheral iNKT cell numbers can recover from a defect in thymic iNKT development indicating the importance of late developmental events occurring post-thymically.

### 3.2.2 DCs trans-present IL-15 to peripheral iNKT cells.

Since IL-15R $\alpha$ <sup>+</sup> hematopoietic cells recover peripheral iNKT cells independent of thymic iNKT cell numbers, the role of DCs as a potential IL-15 trans-presenting cell was investigated. To examine the role of DCs in iNKT cell development and homeostasis, CD11c/IL-15R $\alpha$  transgenic (Tg) mice (on an IL-15R $\alpha$ <sup>-/-</sup> background) were utilized as a model where only DCs can trans-present IL-15<sup>35</sup>. In these CD11c/IL-15R $\alpha$  Tg mice, IL-15R $\alpha$ <sup>+</sup> DCs had little effect in recovering thymic NKT cell numbers leading to only a slight increase over that found in IL-15R $\alpha$ <sup>-/-</sup> mice (**FIGURE 18a and b**).

Interestingly, the loss of CD44<sup>High</sup>NK1.1<sup>+</sup> cells was the major developmental subset affected by the deficiency of IL-15R $\alpha$  (**FIGURE 18c**). In the liver, IL-15R $\alpha$  expression was most crucial compared to the other tissues as its absence resulted in the most dramatic iNKT cell deficiency (<90%) affecting both CD44<sup>High</sup> NK1.1<sup>-</sup> and NK1.1<sup>+</sup> cells (**FIGURE 18b and c**). Moreover, IL-15R $\alpha$ <sup>+</sup> DCs significantly increased the frequency and absolute numbers of total CD1d-tetramer<sup>+</sup>, CD44<sup>High</sup> NK1.1<sup>-</sup>, and CD44<sup>High</sup> NK1.1<sup>+</sup> cells (p<0.05) compared to IL-15R $\alpha$ <sup>-/-</sup> mice (**FIGURE 18a - c**). The frequency and absolute numbers of splenic iNKT cells in Tg mice were increased compared to IL-15R $\alpha$ <sup>-/-</sup> mice but this increase was not significantly different (**FIGURE 18b**). As observed in the chimeras, no preferential effect was observed among either DN or CD4<sup>+</sup> iNKT cell subsets (data not shown). These data demonstrate that iNKT cells respond to DCs specifically in the peripheral

**FIGURE 18. IL-15R $\alpha$ <sup>+</sup> DCs partially recover peripheral iNKT cells.**



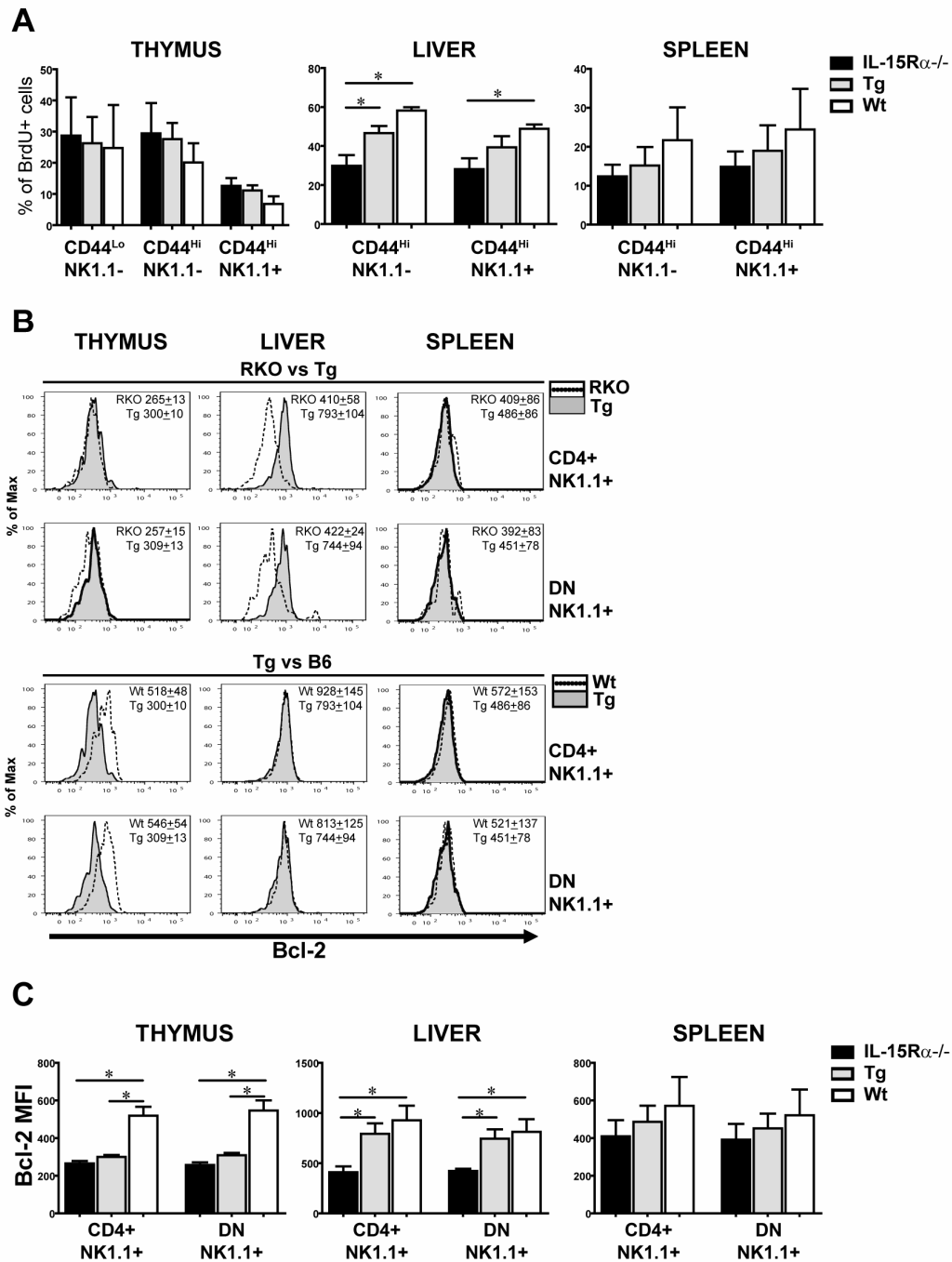
**FIGURE 18. IL-15R $\alpha$ <sup>+</sup> DCs partially recover peripheral iNKT cells. **A**,** Representative flow cytometric plots of CD1d-tetramer<sup>+</sup> CD3<sup>+</sup> cells in the thymus, liver and spleen of IL-15R $\alpha$ <sup>-/-</sup> (left column), CD11c/IL-15R $\alpha$  Tg (middle column) and WT (right column) mice. Plots are representative of three independent experiments. **B**, Absolute numbers of iNKT cells as detected by CD1d-tetramer were calculated for each group of mice, IL-15R $\alpha$ <sup>-/-</sup> (black bar), CD11c/IL-15R $\alpha$  Tg (gray bar) and WT (white bar), in the respective tissues. Error bars represent S.E.M. and the numbers above bars represent percent of WT levels \*  $p \leq 0.05$  (average of 3 independent experiments,  $n = 6$ /group). Tg and WT iNKT cell numbers were statistically compared against total iNKT from IL-15R $\alpha$ <sup>-/-</sup> mice. **C**, graph shows the absolute numbers of iNKT subsets as determined by CD44 and NK1.1 expression in the indicated mice. **"This research was originally published in *Blood* Online. Eliseo F. Castillo, Luis F. Acero, Spencer W. Stonier, Dapeng Zhou, and Kimberly S. Schluns Thymic and peripheral microenvironments differentially mediate development and maturation of iNKT cells by IL-15 transpresentation *Blood* First Edition Paper, prepublished online June 25, 2010;DOI 10.1182/blood-2010-03-277103."**

tissues indicating that thymic and peripheral iNKT cells respond to distinct IL-15R $\alpha$ <sup>+</sup> cells depending on the tissue microenvironment.

### 3.2.3 IL-15R $\alpha$ <sup>+</sup> DCs regulate proliferation and Bcl-2 levels in peripheral iNKT cells.

iNKT cell numbers are dictated in part by proliferation and enhanced survival; therefore these parameters were examined as a means to investigate the mechanism for the increased iNKT cell numbers mediated by IL-15R $\alpha$ <sup>+</sup> DCs. Proliferation is believed to occur simultaneously with differentiation of CD44<sup>Low</sup> NK1.1<sup>-</sup> into CD44<sup>High</sup> NK1.1<sup>-</sup> iNKT cells and the maturation into CD44<sup>High</sup> NK1.1<sup>+</sup> cells<sup>90,107</sup>. To determine whether DCs drive the expansion of iNKT cells via IL-15 trans-presentation, all three groups of mice were treated with BrdU for 7 days and the BrdU incorporation of iNKT cells at the different developmental stages was analyzed (**FIGURE 19a**). Surprisingly, no significant differences in BrdU incorporation by iNKT cells from the three groups of mice were observed at any stage of iNKT cells in the thymus (**FIGURE 19a**). In contrast, BrdU incorporation by hepatic iNKT cells was congruent with the increase in peripheral iNKT cells observed in Tg and WT mice compared to IL-15R $\alpha$ <sup>-/-</sup> mice (**FIGURE 19a**). This effect occurred regardless of NK1.1 expression suggesting that DC-mediated expansion was not restricted to a specific stage of differentiation or maturation (**FIGURE 19a**). Overall, peripheral expansion of hepatic and splenic iNKT cells is mediated by DCs trans-presenting IL-15.

**FIGURE 19. Proliferation and survival of peripheral iNKT cells are supported by DCs via IL-15 trans-presentation.**



**FIGURE 19. Proliferation and survival of peripheral iNKT cells are supported by DCs via IL-15 trans-presentation.**

**A**, graphs depict the percentage of BrdU<sup>+</sup> iNKT subsets in the thymus, liver and spleen of IL-15R $\alpha$ <sup>-/-</sup>, CD11c/IL-15R $\alpha$  Tg, and WT mice after being given BrdU (0.8mg/ml) supplemented drinking water for 7 days. Subsets consisted of the various stages of iNKT cell development as defined by CD44 and NK1.1 expression. Mean  $\pm$  S.E.M. are shown and derived from 2 independent experiments (n=4 for IL-15R $\alpha$ <sup>-/-</sup>, n=5 for CD11c/IL-15R $\alpha$  Tg, n=4 for WT mice, \*p  $\leq$  0.05). **B**, representative histograms comparing the levels of Bcl-2 expression from NK1.1<sup>+</sup> subsets derived IL-15R $\alpha$ <sup>-/-</sup> and CD11c/IL-15R $\alpha$  Tg mice (top two panels) or CD11c/IL-15R $\alpha$  Tg and WT mice (bottom two panels) in the various tissues. **C**, the graph displays the mean fluorescence intensity of Bcl-2 from CD4<sup>+</sup> or DN NK1.1<sup>+</sup> subsets isolated from the thymus, liver and spleen of IL-15R $\alpha$ <sup>-/-</sup>, CD11c/IL-15R $\alpha$  Tg, WT mice. Mean  $\pm$  S.E.M are shown and derived from 3 independent experiments (n= 6-8 mice/group, \*p  $\leq$  0.05). "This research was originally published in *Blood* Online. Eliseo F. Castillo, Luis F. Acero, Spencer W. Stonier, Dapeng Zhou, and Kimberly S. Schluns Thymic and peripheral microenvironments differentially mediate development and maturation of iNKT cells by IL-15 transpresentation *Blood* First Edition Paper, prepublished online June 25, 2010;DOI 10.1182/blood-2010-03-277103."

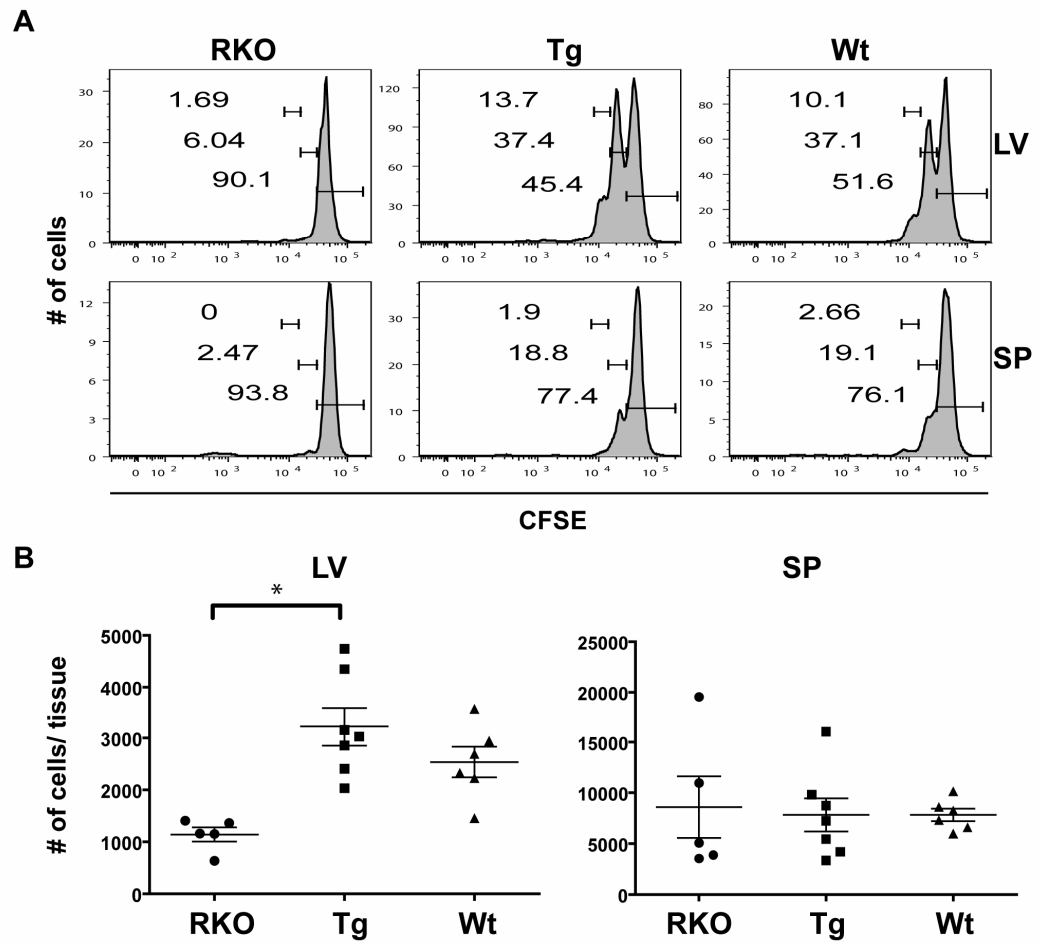


IL-15 up-regulates Bcl-2 expression and is one mechanism by which it increases cell survival<sup>136</sup>. In iNKT cells, Bcl-2 expression is also increased upon acquisition of NK1.1<sup>+</sup> expression<sup>110</sup>. To determine whether DC-mediated increases in iNKT cells correlated to Bcl-2 levels, Bcl-2 expression was examined in iNKT cells by quantitating the mean fluorescence intensity (MFI) of Bcl-2 via flow cytometry. In thymic iNKT cells, both CD4<sup>+</sup> or DN NK1.1<sup>+</sup> cells from WT mice expressed significantly higher levels of Bcl-2 than the analogous subsets from either IL-15R $\alpha$ <sup>-/-</sup> and Tg mice (**FIGURE 19b and c**). In the liver, Bcl-2 expression in CD4<sup>+</sup> and DN iNKT cells from the Tg mice was significantly higher ( $p < 0.05$ ) than the same cells from IL-15R $\alpha$ <sup>-/-</sup> mice and was quite comparable to that seen in WT mice (**FIGURE 19b and c**). In splenic iNKT cells, Bcl-2 had a hierarchal expression among IL-15R $\alpha$ <sup>-/-</sup> < Tg < WT mice; however, this trend was not statistically significant (**FIGURE 19b and c**). In general, these findings suggest that reduced numbers of iNKT cells observed in the thymus of IL-15R $\alpha$ <sup>-/-</sup> and Tg mice are due to decrease numbers of CD44<sup>High</sup> NK1.1<sup>+</sup> iNKT cells resulting in part by reduced Bcl-2 but not defective proliferation. In the periphery, particularly in the liver, the partial restoration of iNKT cells in the Tg mice is mediated by DCs trans-presenting IL-15, which affect both the proliferation and survival of iNKT cells during late development.

### 3.2.4 Homeostatic proliferation of iNKT cells is mediated by DCs via IL-15 trans-presentation.

Similar to memory CD8 T cells, the homeostasis of mature iNKT cells is mediated by basal proliferation and is similarly dependent on IL-15<sup>34,111</sup>; however, the cell-type mediating this event for iNKT cells has not been elucidated. To assess the role of IL-15R $\alpha$ <sup>+</sup> DCs in regulating iNKT cell homeostasis, mature iNKT cells (>94% CD44<sup>High</sup>NK1.1<sup>+</sup>) were enriched from the thymus of WT mice (CD45.1<sup>+</sup>), CFSE-labeled, and transferred to IL-15R $\alpha$ <sup>-/-</sup>, Tg, and WT mice (all CD45.2<sup>+</sup>). Seven days after transfer, donor-derived CD1d-tetramer<sup>+</sup> cells were detected in the spleen and liver, but not the thymus of all mice. Among the different tissues, the liver was the major site of iNKT cell homeostatic proliferation. As expected, very little cell division of iNKT cells was observed upon transfer into IL-15R $\alpha$ <sup>-/-</sup> hosts while a low level of proliferation was observed in WT hosts (**FIGURE 20a**). Upon transfer into Tg mice, iNKT cells underwent a similar level of proliferation as that in WT hosts indicating that the presence of IL-15R $\alpha$ <sup>+</sup> DCs was sufficient for iNKT cell proliferation (**FIGURE 20a**). Furthermore, the number of donor iNKT cells found in the liver was completely restored by the presence of IL-15R $\alpha$ <sup>+</sup> DCs while decreased in IL-15R $\alpha$ <sup>-/-</sup> mice (**FIGURE 20b**). Interestingly, the maintenance of iNKT cells in the spleen did not depend on IL-15R $\alpha$  expression (**FIGURE 20b**). These findings demonstrate that DCs are a major cell-type providing IL-15 in the liver for homeostatic proliferation.

**FIGURE 20. IL-15R $\alpha$ <sup>+</sup> DCs drive the homeostatic proliferation of mature iNKT cells.**

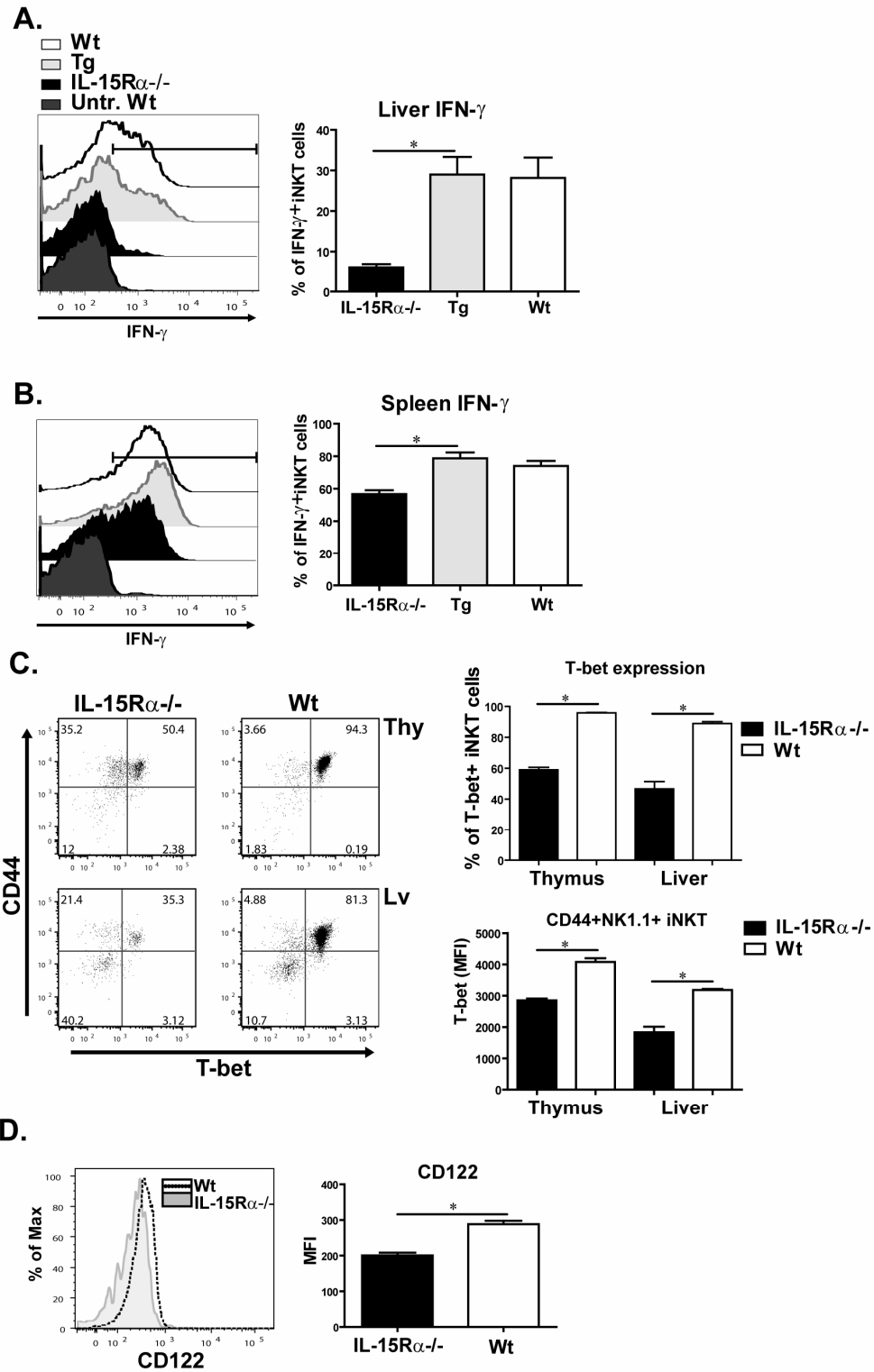


**FIGURE 20. IL-15R $\alpha$ <sup>+</sup> DCs drive the homeostatic proliferation of mature iNKT cells.** iNKT cells enriched from the thymus of WT congenic (CD45.1) mice were labeled with CFSE and then transferred to congenic (CD45.2) IL-15R $\alpha$ <sup>-/-</sup>, CD11c/IL-15R $\alpha$  Tg and WT mice. Seven days after the transfer, mice were sacrificed to assess the recovery and CFSE dilution of donor cells in the various tissues of the three groups of mice. **A**, dilution of CFSE by donor iNKT cells (CD45.1<sup>+</sup>) found in the spleen and liver seven days after the adoptive transfer into congenic CD45.2<sup>+</sup> IL-15R $\alpha$ <sup>-/-</sup>, CD11c/IL-15R $\alpha$  Tg and WT mice. **B**, graph shows the absolute number of donor iNKT cells (gated on CD45.1<sup>+</sup> CD1d-tetramer<sup>+</sup> cells) recovered in the liver and spleen seven days after the adoptive transfer into the three groups of mice. Horizontal bar is the average of 3 independent experiments (n=5-7 mice/group, \* p<0.001). **"This research was originally published in *Blood* Online. Eliseo F. Castillo, Luis F. Acero, Spencer W. Stonier, Dapeng Zhou, and Kimberly S. Schluns Thymic and peripheral microenvironments differentially mediate development and maturation of iNKT cells by IL-15 transpresentation *Blood* First Edition Paper, prepublished online June 25, 2010;DOI 10.1182/blood-2010-03-277103."**

### 3.2.5 IL-15 is important for functional maturation of IFN- $\gamma$ producing hepatic iNKT cells.

A distinguishing feature of iNKT cells is their ability to rapidly produce IFN- $\gamma$  upon stimulation with  $\alpha$ GalCer<sup>87</sup>. Therefore, to assess the functional responsiveness of iNKT cells *in vivo*, IFN- $\gamma$  expression by iNKT cells was analyzed 2.5 hrs after i.v injection of  $\alpha$ GalCer in the various mice (IL-15R $\alpha^{-/-}$ , Tg, and WT). Previous studies have reported that thymic iNKT cells in normal mice do not express IFN- $\gamma$  in response to  $\alpha$ GalCer<sup>87</sup>. This finding was also found to be true in my hands (data not shown). In the liver, few iNKT cells produced IFN- $\gamma$  in IL-15R $\alpha^{-/-}$  mice while a significant proportion of iNKT cells expressed IFN- $\gamma$  in response to  $\alpha$ GalCer in Tg and WT mice (**FIGURE 21a and b**). Interestingly, iNKT cells in the spleen of all three groups of mice produced IFN- $\gamma$  after  $\alpha$ GalCer stimulation, albeit, the percent of iNKT cells producing IFN- $\gamma$  in IL-15R $\alpha^{-/-}$  mice was significantly reduced compared to WT and Tg mice (**FIGURE 21b**). This finding demonstrates that functional maturation of splenic iNKT cells is not heavily dependent on IL-15R $\alpha$  and therefore may be mediated by other factors. IL-4 was also expressed by iNKT cells at low levels after  $\alpha$ GalCer stimulation but was not affected by IL-15R $\alpha$  expression (data not shown). Nevertheless, functional maturation of hepatic iNKT cells is critically dependent on IL-15 signaling, which can be provided by DCs.

**FIGURE 21. IL-15 signaling optimizes T-bet induced gene products in iNKT cells.**



**FIGURE 21. IL-15 signaling optimizes T-bet induced gene products in iNKT cells.** iNKT cells were activated by administering  $\alpha$ GalCer to each group of mice. iNKT cells from the liver and spleen were analyzed for IFN- $\gamma$  production (2.5 hrs later) via intracellular cytokine staining. Representative histograms showing the percent of hepatic (**A**) and splenic (**B**) iNKT cells producing IFN- $\gamma$  after  $\alpha$ GalCer administration from treated mice (IL-15R $\alpha^{-/-}$ , Tg, and WT mice) and untreated WT mice (negative control). The graph shows the average percent of IFN- $\gamma^{+}$  iNKT cells from the liver (**A**) and spleen (**B**) from all treated groups. Error bars represent S.E.M. and is combined data from two independent experiments, n= 4-5 mice/group, \*p  $\leq$  0.005. **C**, flow cytometric plots displaying CD44 and T-bet expression in CD1d-tetramer $^{+}$  cells from the thymus and liver of IL-15R $\alpha^{-/-}$  and WT mice. Upper right graph shows the percentage of T-bet $^{+}$  iNKT cells from the thymus and liver of the two groups (\*p<0.0005). Lower right graph shows average MFI of T-bet expression in CD44 $^{High}$ NK1.1 $^{+}$  iNKT cells from IL-15R $\alpha^{-/-}$  and WT mice isolated from the thymus and liver (\*p<0.0001). **D**, representative histogram of CD122 expression in thymic CD44 $^{+}$ NK1.1 $^{+}$  iNKT cells from IL-15R $\alpha^{-/-}$  and WT mice and the graph displays the average MFI of CD122 expression from both groups (\*p<0.0001). Fig 4C-D combined data from two independent experiments, n=6 for both IL-15R $\alpha^{-/-}$  and WT mice. Error bars represent S.E.M. **"This research was originally published in *Blood* Online. E.F. Castillo et al., Thymic and peripheral microenvironments differentially mediate development and maturation of iNKT cells by IL-15 transpresentation. *Blood* First Edition Paper, prepublished online June 25, 2010;DOI 10.1182/blood-2010-03-277103."**

The low level of IFN- $\gamma$  expression in IL-15R $\alpha^{-/-}$  iNKT cells after  $\alpha$ GalCer stimulation suggests these cells are functionally immature or unprimed. The transcription factor, T-bet, drives the functional maturation of iNKT cells by regulating the expression of several genes including CD122 and IFN- $\gamma$ <sup>59,116</sup>. Therefore, T-bet expression was analyzed in iNKT cells from both IL-15R $\alpha^{-/-}$  and WT mice via flow cytometry. Since iNKT cells in thymus and liver are heavily dependent on IL-15R $\alpha$ , we specifically analyzed iNKT cells from these two tissues (**FIGURE 21c**). Although T-bet was expressed by IL-15R $\alpha^{-/-}$  iNKT cells, only 59% and 47% of the population expressed high levels of T-bet in the thymus and liver, respectively, which was significantly less than the observed among iNKT cells in the thymus (96%) and liver (89%) of WT mice (**FIGURE 21c**). High T-bet expression was relevant as IFN- $\gamma$  expression was restricted to this population (data not shown). Among CD44<sup>High</sup> NK1.1<sup>+</sup> iNKT cells, the level of T-bet expression was significantly reduced in both the thymus and liver, but not spleen, of IL-15R $\alpha^{-/-}$  mice indicating the overall, reduced T-bet was not just due to disproportional subsets of developing iNKT cells (**FIGURE 21c**, data not shown). In addition to T-bet, CD122 expression by thymic CD44<sup>High</sup> NK1.1<sup>+</sup> iNKT cells was notably reduced in IL-15R $\alpha^{-/-}$  mice (**FIGURE 21d**). Although T-bet and CD122 are both reduced in the absence of IL-15R $\alpha$ , double staining for CD122 and T-bet was unable to directly demonstrate a correlation in expression, which may be an indication that T-bet is not the only regulator of CD122. Thus, the reduced number of IFN- $\gamma$ <sup>+</sup> iNKT cells responding to  $\alpha$ GalCer stimulation in IL-15R $\alpha^{-/-}$  mice suggests



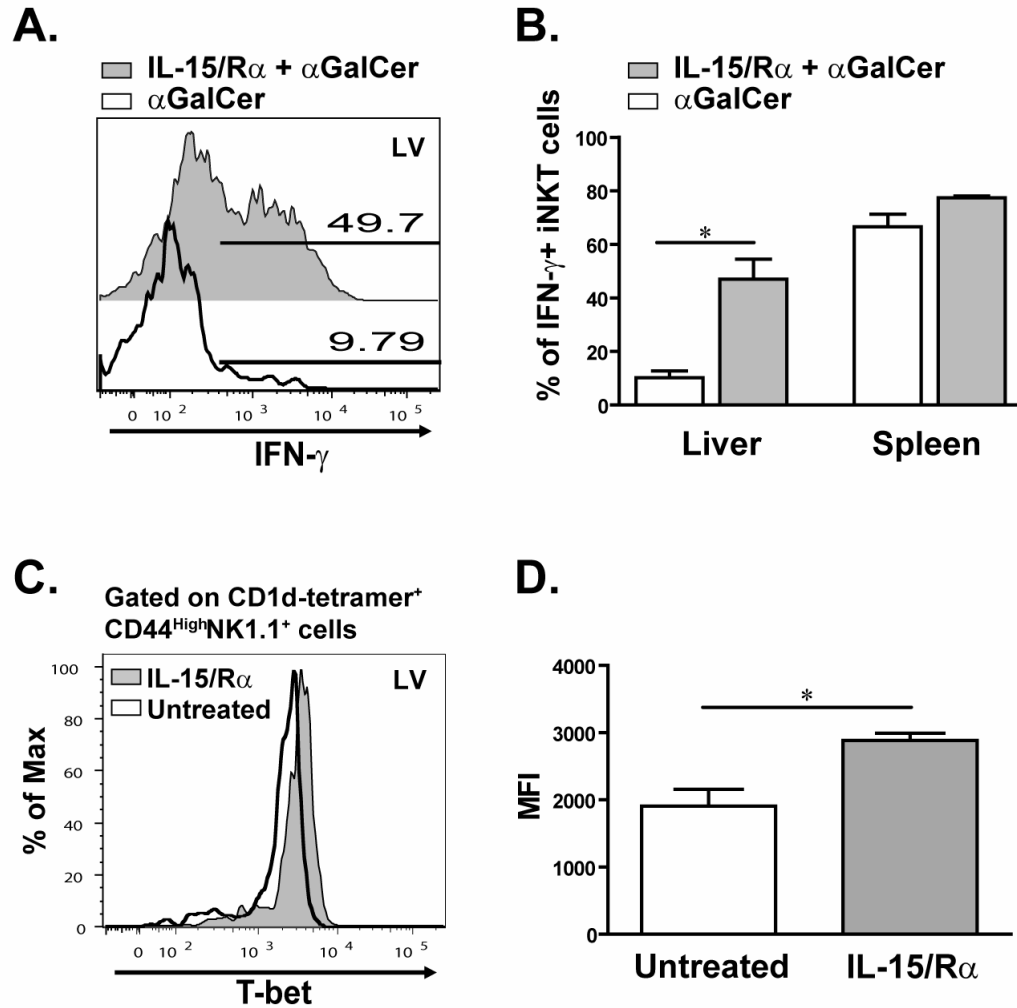
these iNKT cells are not fully functional, which could be due in part to the lack of IL-15-mediated expression of T-bet and/or CD122.

### 3.2.6 IL-15/IL-15R $\alpha$ complex enhances IFN- $\gamma$ and T-bet expression in IL-15R $\alpha$ <sup>-/-</sup> iNKT cells.

The above observations suggest IL-15 contributes to the functional maturation of iNKT cells. To determine if IL-15 signaling could recover the defect observed in the absence of IL-15R $\alpha$ , IL-15R $\alpha$ <sup>-/-</sup> mice were treated with IL-15/IL-15R $\alpha$  complex. Past studies have shown IL-15/IL-15R $\alpha$  complex provides a potent IL-15 signal independent of trans-presentation<sup>137</sup> and does not influence the production of IFN- $\gamma$  by iNKT cells<sup>138</sup>. Since the activity of the complex declines considerably within 24 hrs<sup>137</sup>, possible effects of IL-15 stimulation on maturation can be separated from those during activation by stimulating mice with  $\alpha$ GalCer 2 days after the initial IL-15 treatment.

Accordingly, IL-15R $\alpha$ <sup>-/-</sup> mice were pretreated with the IL-15/IL-15R $\alpha$  complex, then injected i.v. with  $\alpha$ GalCer followed by analysis of IFN- $\gamma$  expression 2.5 hrs later. Interestingly, pretreatment with IL-15/IL-15R $\alpha$  significantly amplified the percentage of hepatic iNKT cells expressing IFN- $\gamma$  in response to  $\alpha$ GalCer compared to those iNKT cells in IL-15R $\alpha$ <sup>-/-</sup> mice not receiving a prior IL-15 signal (**FIGURE 22a**). Pretreatment with IL-15/IL-15R $\alpha$  also increased the expression of IFN- $\gamma$  in splenic iNKT cells; however this effect was not statistically significant (**FIGURE 22b**). This recovery in IFN- $\gamma$  expression by

**FIGURE 22. Pretreatment with IL-15/IL-15R $\alpha$  complex restores iNKT cell responses in IL-15R $\alpha$ <sup>-/-</sup> mice.**



**FIGURE 22. Pretreatment with IL-15/IL-15R $\alpha$  complex restores iNKT cell responses in IL-15R $\alpha$ <sup>-/-</sup> mice.** Analysis of iNKT cells producing IFN- $\gamma$  after  $\alpha$ GalCer administration in IL-15R $\alpha$ <sup>-/-</sup> mice pretreated with or without IL-15/IL-15R $\alpha$  complex. **A**, representative histogram overlaying the percent of hepatic iNKT cells producing IFN- $\gamma$  from treated and untreated IL-15R $\alpha$ <sup>-/-</sup> mice. **B**, graph shows the average percent of iNKT cells in the liver and spleen expressing IFN- $\gamma$  in response to  $\alpha$ GalCer in mice either pretreated or untreated with IL-15/IL-15R $\alpha$  complex. **C and D**, effect of IL-15 signaling on T-bet expression in IL-15R $\alpha$  deficient mice. IL-15/IL-15R $\alpha$  complex was injected into IL-15R $\alpha$ <sup>-/-</sup> mice and one day later, T-bet expression in iNKT cells was analyzed by flow cytometry. **C**, representative histogram displaying an overlay of the level of T-bet expressed in CD1d-tetramer<sup>+</sup> CD44<sup>High</sup>NK1.1<sup>+</sup> cells isolated from liver of treated and untreated IL-15R $\alpha$ <sup>-/-</sup> mice. **D**, combined data presenting the average MFI of T-bet in CD44<sup>High</sup>NK1.1<sup>+</sup> iNKT cells from the liver of IL-15/IL-15R $\alpha$  complex treated or untreated mice. Graphs shown in B and D are generated from two independent experiments, n=4 mice. Error bars represent S.E.M., \*p<0.05. **"This research was originally published in *Blood* Online. Eliseo F. Castillo, Luis F. Acero, Spencer W. Stonier, Dapeng Zhou, and Kimberly S. Schluns Thymic and peripheral microenvironments differentially mediate development and maturation of iNKT cells by IL-15 transpresentation *Blood* First Edition Paper, prepublished online June 25, 2010;DOI 10.1182/blood-2010-03-277103."**

IL-15/IL-15R $\alpha$  pretreatment supports the notion that IL-15 primes hepatic iNKT cells for IFN- $\gamma$  production.

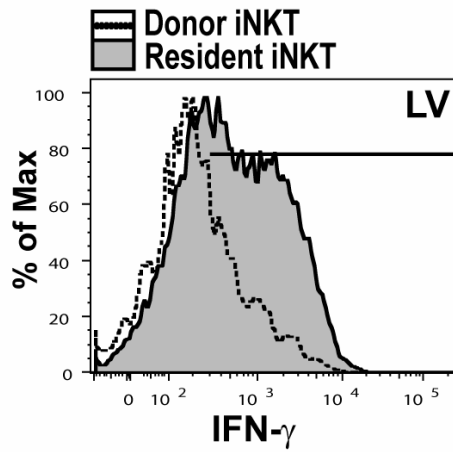
To determine if the IL-15 signals affect the key regulator in iNKT functional maturation, T-bet was analyzed in IL-15R $\alpha$ <sup>-/-</sup> mice treated with just the IL-15/IL-15R $\alpha$  complex. A day after injection of the IL-15/IL-15R $\alpha$  complex, hepatic CD44<sup>High</sup> NK1.1<sup>+</sup> iNKT cells had a significant increase in T-bet expression when compared to the corresponding population in untreated IL-15R $\alpha$ <sup>-/-</sup> mice (**FIGURE 22c and d**). So, although T-bet expression is low in iNKT cells in IL-15R $\alpha$ <sup>-/-</sup> mice, encountering IL-15 in vivo increases the level of T-bet expression in mature iNKT cells. Collectively, this data demonstrates IL-15 signaling not only boost iNKT cells ability to produce IFN- $\gamma$  in response to  $\alpha$ GalCer but also augments T-bet expression.

### 3.2.7 IL-15 trans-presentation in periphery promotes functional responsiveness in vivo.

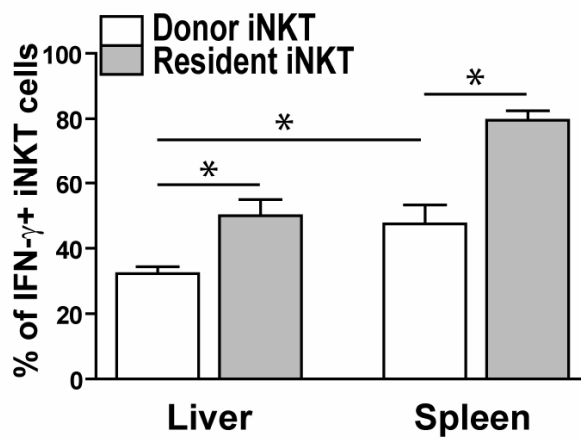
As previously mentioned, thymic iNKT do not respond to  $\alpha$ GalCer in vivo<sup>87</sup>; however, thymic and hepatic iNKT cells express IFN- $\gamma$  to a similar degree when stimulated with  $\alpha$ GalCer in the presence of DCs<sup>103</sup>. Therefore, we asked if thymic iNKT cells become functionally responsive in a different in vivo tissue microenvironment and whether this is dependent on IL-15R $\alpha$  expression. Therefore, iNKT cells were enriched from the thymus of WT (CD45.1) mice, adoptively transferred into WT or IL-15R $\alpha$ <sup>-/-</sup> (CD45.2) mice

**FIGURE 23. Thymic iNKT cells respond to  $\alpha$ GalCer in the periphery in the presence of IL-15R $\alpha$ .**

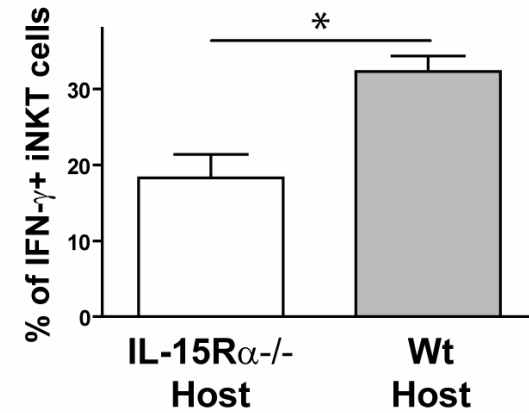
**A.**



**B.**



**C.**



**FIGURE 23. Thymic iNKT cells respond to  $\alpha$ GalCer in the periphery in the presence of IL-15R $\alpha$ .** iNKT cells were enriched from the thymus of congenic (CD45.1) WT mice and transferred into a congenic (CD45.2) WT host to seed the periphery. Two days after the cell transfer, mice were injected with  $\alpha$ GalCer to activate iNKT cells *in vivo*. **A**, Representative histogram showing the level of IFN- $\gamma$  being produced by donor (open histogram) and resident iNKT cells (shaded histogram) in liver of mice which received  $\alpha$ GalCer two days after the adoptive transfer. **B**, bar graph of the combined data of IFN- $\gamma$ <sup>+</sup> donor (white bars) and resident (gray bars) iNKT cells isolated from the liver or spleen of mice stimulated with  $\alpha$ GalCer *in vivo*. Data is generated from two independent experiments (n=4), **C**, bar graph of the combined data of donor IFN- $\gamma$ <sup>+</sup> iNKT cells isolated from the liver of IL-15R $\alpha$ <sup>-/-</sup> (white bar) and WT (gray bars) mice. Data are generated from three independent experiments (n=6 for IL-15R $\alpha$ <sup>-/-</sup> host and n=4 for WT host). Error bars represent S.E.M., \*p<0.05. **"This research was originally published in *Blood* Online. Eliseo F. Castillo, Luis F. Acero, Spencer W. Stonier, Dapeng Zhou, and Kimberly S. Schluns Thymic and peripheral microenvironments differentially mediate development and maturation of iNKT cells by IL-15 transpresentation *Blood* First Edition Paper, prepublished online June 25, 2010;DOI 10.1182/blood-2010-03-277103."**

and then analyzed for IFN- $\gamma$  expression in response to  $\alpha$ GalCer. In WT mice, donor thymic iNKT cells became responsive to  $\alpha$ GalCer in the liver and spleen; however, the donor iNKT cells were not as responsive as the resident iNKT cells (**FIGURE 23a and b**). In addition, IFN- $\gamma$  expression by donor iNKT cells was increased in the spleen compared to the liver (**FIGURE 23a and b**). In the absence of host IL-15R $\alpha$ , the percent of iNKT cells expressing IFN- $\gamma$  in response to  $\alpha$ GalCer was further decreased to a significant degree (**FIGURE 23c**) suggesting IFN- $\gamma$  responses in the liver were dependent on IL-15R $\alpha$ . Collectively, these results suggest that encountering IL-15 in the periphery promotes and enhances the ability of iNKT cells to express IFN- $\gamma$  in response to stimulation, thus highlighting an important role for IL-15 in iNKT cell maturation and activation.

### 3.3 Discussion

Preferences in cell-specific IL-15 trans-presentation by hematopoietic or non-hematopoietic cells have been shown for memory CD8<sup>+</sup> T cells, NK cells, and iIELs, but not for iNKT cells<sup>29,135</sup>. Moreover, while IL-15 has a clear role in iNKT cell development and homeostasis<sup>13,34,111</sup>, the role of IL-15 trans-presentation in these processes had not been previously investigated. Herein, I show the cell-type trans-presenting IL-15 to iNKT cells as well as the function of IL-15 was dependent on the tissue site. Furthermore, I revealed a new role for IL-15 in the functional maturation and activation of iNKT cells. Taken together, my work highlights how IL-15 regulates the development, functional maturation, and homeostasis of iNKT cells through multiple mechanisms, which are mediated by distinct cell types in a tissue-specific fashion.

IL-15 is capable of mediating differentiation, proliferation, and survival; however, the role of IL-15 on iNKT cells was different between tissues. In the thymus, IL-15 trans-presentation was essential for survival but not so much for the expansion of developing iNKT cells. Other studies have found CD28/B7 and ICOS/B7h co-stimulation drives intrathymic iNKT cell expansion<sup>139</sup>. Thus, the proliferation observed in thymus of IL-15R $\alpha$ -deficient mice could be due to co-stimulation and other  $\gamma$ C cytokines. In contrast to the thymus, IL-15 influenced both the proliferation and survival of iNKT cells in the periphery. My finding of IL-15-mediated proliferation is in



contrast to the lack of proliferation of iNKT cells observed in IL-15 Tg mice<sup>140</sup>. In this IL-15 Tg model, the excess IL-15 produced likely binds to the many cell types expressing IL-15R $\alpha$ , which do not normally trans-present IL-15. Therefore, situations of excess IL-15 may yield different outcomes because IL-15 is not provided by specific cell types. I provide evidence that IL-15 regulates cell survival as peripheral iNKT cells displayed reduced levels of Bcl-2 and were not sustained in IL-15R $\alpha$ <sup>-/-</sup> mice. While IL-15 notoriously drives proliferation of mature CD44<sup>High</sup> NK1.1<sup>+</sup> cells<sup>34</sup>, I found the presence of IL-15R $\alpha$  also increased the proliferation of immature hepatic CD44<sup>High</sup> NK1.1<sup>-</sup> cells. Since proliferation of immature CD44<sup>High</sup> NK1.1<sup>-</sup> cells was modulated by IL-15 in the liver but not in the thymus, it suggests that the type of IL-15 response is better dictated by the microenvironment than the stage of differentiation.

In contrast to the thymus and liver, splenic iNKT cells in IL-15R $\alpha$ <sup>-/-</sup> mice were only minimally deficient in numbers and responded well to  $\alpha$ GalCer. Altogether, these findings could suggest that another factor is substituting for IL-15 in the spleen. As IL-7 shares many of the same signals as IL-15 and has been shown to be important for splenic iNKT cells<sup>34</sup>, I think IL-7 is a good candidate. Interestingly, as IL-15 is provided to other cell types in the spleen, it is unclear why splenic iNKT cells do not assess these IL-15-producing cells. I speculate other mechanisms determine the niche where specific cells reside in or mediate specific cell-cell interactions. Alternatively,

iNKT cells in the spleen and liver may be distinct populations that have obtained differential requirements for the respective cytokines.

It is often assumed that the function of IL-15 in lymphocyte development and homeostasis is to induce survival and proliferation; however, my analyses of iNKT cell responses provide evidence that the functions of IL-15 go beyond regulating cell numbers. Somewhere along the developmental pathway, iNKT cells acquire the ability to rapidly respond to TCR stimulation by producing both IL-4 and IFN- $\gamma$ . As IL-4 and IFN- $\gamma$  expression are regulated by Gata-3 and T-bet, respectively, the expression of these transcription factors is important for functional maturation of iNKT cells <sup>116</sup>. Therefore, it was interesting to find that T-bet expression was decreased in iNKT cells in IL-15R $\alpha^{-/-}$  mice. While this could be due to a preferential loss of T-bet<sup>+</sup> cells, T-bet was up-regulated in a short term response to IL-15.

More importantly, the ability of iNKT cells to express IFN- $\gamma$  in response to  $\alpha$ GalCer was virtually lost in IL-15R $\alpha^{-/-}$  mice but restored by prior exposure to IL-15. In Th1 cells, signaling through the  $\gamma$ C subunit via IL-2 revealed the JAK3/STAT5 pathway is required for IFN- $\gamma$  production <sup>141</sup>. Essentially, STAT5 grants T-bet access to the IFN- $\gamma$  promoter region by promoting chromatin accessibility. As IL-15 and IL-2 both utilize the same receptor subunits and signaling (JAK3/STAT5) pathway, IL-15 could be the physiological factor activating STAT5 in iNKT cells rather than IL-2.

Additionally, since repetitive administration with  $\alpha$ GalCer impairs IFN- $\gamma$  production in iNKT cells<sup>142,143</sup>, it would be interesting to investigate whether IL-15/IL-15R $\alpha$  treatment can reverse this defect. Overall, my findings suggest that IL-15 is important for promoting IFN- $\gamma$  production in iNKT cells and therefore treatment of IL-15/IL-15R $\alpha$  prior to  $\alpha$ GalCer might generate a more potent IFN- $\gamma$  response.

I noted the function of IL-15 during iNKT cell development varied in different tissue microenvironment, which may be credited to the cell-type trans-presenting IL-15. DCs, macrophages, and thymic stromal cells are all present and capable of trans-presenting IL-15 in the thymus; however, despite this, thymic iNKT cells respond only to IL-15 in the non-hematopoietic compartment, again supporting the idea that the niches or cell-cell interactions are tightly regulated. Medullary thymic epithelial cells are likely the non-hematopoietic cell-type trans-presenting IL-15 to iNKT cells as prior studies found that RelB-deficient mice lack normal medullary thymic epithelial cells, have defective iNKT cell development, and reduced levels of IL-15 transcript<sup>119,120</sup>. Surprisingly, despite our finding that non-hematopoietic expression of IL-15R $\alpha$  was completely sufficient to restore iNKT cell numbers in the thymus, a deficiency in iNKT cells in the liver was still apparent.

Moreover, a complete deficiency of iNKT cells in the thymus did not translate to a major deficiency in either the liver or the spleen. This demonstrates the

unappreciated importance of post-thymic development and maturation and illustrates the dependence on the peripheral microenvironment for completing development and maintaining mature iNKT cells.

Unlike thymic iNKT cells, peripheral iNKT cells receive IL-15 trans-presented by either hematopoietic or non-hematopoietic cells. Here, I identify DCs as a major cell-type regulating Bcl-2 and mediating homeostatic proliferation while having less of an impact on total cell numbers; this could be indicative that DCs have a more minor role in late post-thymic expansion and more important role in homeostasis. More surprisingly, DCs were able to restore functional maturation of iNKT cells in the liver, thus identifying a new role for DCs in iNKT cell biology. This ability to induce IFN- $\gamma$  expression in iNKT cells may provide another mechanism by which DCs dictate the fate of an immune response. IL-15R $\alpha$  BM chimeras also revealed hepatic iNKT cell numbers were controlled by IL-15R $\alpha^+$  non-hematopoietic cells, which are likely hepatic stellate cells (Ito cells) <sup>121</sup>. It would be interesting to determine whether Ito cells are also capable of promoting functional maturation.

Regarding IL-15R $\alpha$  expression by iNKT cells, a recent study revealed RelA/NF- $\kappa$ B controls IL-15 signaling in iNKT cells by enhancing IL-15R $\alpha$  expression in iNKT cells <sup>117</sup>. In support of iNKT cells responding to IL-15 through the IL-15R $\alpha\beta\gamma$  heterotrimeric complex, IL-15R $\alpha^{-/-}$  mice lack iNKT cells <sup>21</sup>. Thus, the phenotype of IL-15R $\alpha^{-/-}$  mice and the role of RelA in IL-

15R $\alpha$  expression makes it plausible that iNKT cells can respond to IL-15 through the IL-15R $\alpha\beta\gamma$  heterotrimeric complex. Although this hypothesis is possible, my data using BM chimeras demonstrates that IL-15R $\alpha$  expression by iNKT cells is unnecessary for the generation of iNKT cells. Future work should address if IL-15R $\alpha$  expression by iNKT cells is linked to Axl or NKG2D signaling as these molecules have been shown to associate with IL-15R $\alpha$  or the IL-15 signaling pathway, respectively<sup>144,145 „ 146</sup>.

In conclusion, I have identified the cell-types regulating the development and homeostasis of iNKT cells via IL-15 trans-presentation in the various tissue microenvironments where iNKT cell reside.

## CHAPTER 4

### CONCLUSIONS AND DISCUSSION

#### 4.1 Summary of Chapter 2.

##### 4.1.1 The source of IL-15 during NK cell development and homeostasis.

Prior studies investigating the role of IL-15R $\alpha$  expression by hematopoietic and non-hematopoietic cells provided the first clues that multiple diverse cell-types could trans-present IL-15 to NK cells <sup>29</sup>. My data support these prior reports and extends on these findings by revealing both compartments trans-present IL-15 but at different developmental stages. Thus, the maturation status of NK cells dictates the cell-type trans-presenting IL-15. Also, it was previously thought non-hematopoietic cells were the primary source of IL-15; however, this report reveals for the first time the hematopoietic compartment is the major source of IL-15 during NK cell development and homeostasis.

NK cells become dependent on IL-15 early during development. The earliest population committed to the NK cell lineage is NKp's; however, this CD122-expressing population is not dependent on IL-15. The transition of NKp's to immature NK cells marks their dependency on IL-15 <sup>48</sup>. By actually examining the NK cell stages recovered by the various cellular IL-15R $\alpha$ <sup>+</sup> compartments, I find this only partially true. Immature NK cells can be separated into two states based on Ly49 expression. The Ly49<sup>-</sup> population, like NKp's, develops in the absence of IL-

15. Upon the acquisition of Ly49 receptors, NK cells become reliant on IL-15. In the BM and liver, both cellular compartments are equally important in providing IL-15 at this stage of development. In the spleen, the hematopoietic compartment is the dominant source of IL-15 to generate this population. Interestingly, IL-15 trans-presentation by the hematopoietic compartment emerges as the more efficient population to drive Ly49 expression by NK cells <sup>67</sup>.

The transition to become functionally mature NK cells comes after acquiring the various Ly49 activating and inhibitory receptors. Like immature counterparts, mature NK cells can be separated into two separate stages based on DX5 and CD11b expression. The M1 (DX5+ CD11b<sup>Low</sup>) population is found in the BM, LV, and SP and the presence of M1 NK cells in each of these tissues is contingent on hematopoietic cells providing IL-15. The M2 (DX5+ CD11b<sup>High</sup>) NK cells, which are derived from M1 NK cells, are even more dependent on IL-15 trans-presentation by hematopoietic cells in each of these tissues. Although, the non-hematopoietic compartment can partially recover both populations of mature NK cells, mature NK cells are more likely to interact with hematopoietic cells at this stage of development.

This “division of labor” by both cellular compartments is likely due to the cell-cell interaction and the exact location of NK cells in the tissue microenvironment. It could be envisioned NKp’s and Ly49<sup>-</sup> immature NK cells are in the same location as BM stroma where they begin to see IL-15. In this niche, NKp’s differentiate into

Ly49<sup>-</sup> NK cells through as yet undetermined mechanism that is independent of IL-15. Additionally, the Ly49<sup>-</sup> NK cells respond to IL-15 and differentiate into Ly49<sup>+</sup> NK cells. This population is dependent on IL-15 and probably vacates the stromal niche to acquire IL-15 from a different cellular source (i.e. hematopoietic cells) and to provide space for progenitor cells (i.e. NKp's and Ly49<sup>-</sup> NK cells). Once out of the BM stroma niche, NK cell further differentiate by responding to hematopoietic cell-mediated IL-15 trans-presentation. This "division of labor" model is depicted in **FIGURE 24**. These same events likely occur in the liver given that this tissue microenvironment contains cells from both cellular compartments capable of producing IL-15. On the contrary, the spleen contains far more hematopoietic cells than non-hematopoietic cells; hence, a more dominant requirement of IL-15 being provided by hematopoietic cells for splenic NK cells. In conclusion, the cell-type trans-presenting IL-15 is dependent on the composition of cells in the tissue site and the level of NK cell maturity.

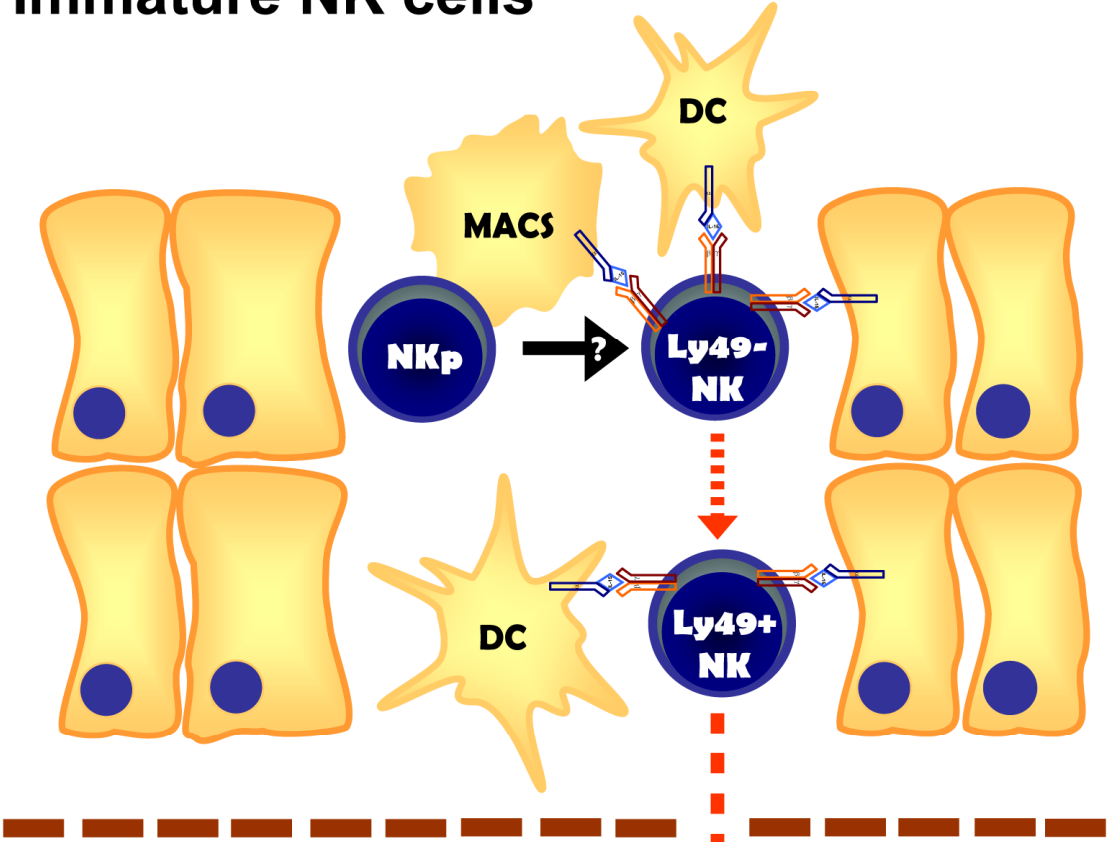
#### 4.1.2 The function of IL-15 during NK cell development and homeostasis.

IL-15 is known to influence multiple cellular functions including proliferation and survival. Past reports have shown IL-15 is crucial for NK cell survival by regulating the anti-apoptotic molecules, Bcl-2 and Mcl-1<sup>69,72</sup>. IL-15 can also expand NK cells in vitro; however, the primary in vivo role of IL-15 during NK cell development and homeostasis is thought to mediate survival (via Bcl-2 and Mcl-1) since the adoptive transfer of CFSE-labeled NK cells into IL-15<sup>-/-</sup> and WT mice have a similar CFSE

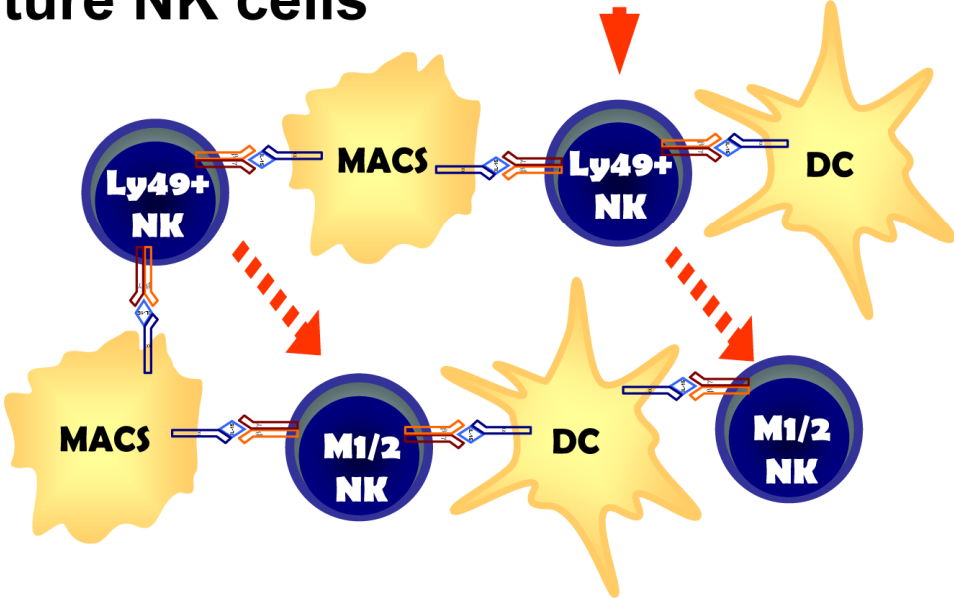


FIGURE 24. Regulation of NK cell development.

Immature NK cells



Mature NK cells



**FIGURE 24. Regulation of NK cell development.**

An illustrative model is shown depicting the various hematopoietic and non-hematopoietic cell-types trans-presenting IL-15 to immature and mature NK cells. Briefly, NKp's and Ly49<sup>-</sup> NK cells (both IL-15-independent populations) are surrounded by the BM stromal niche. Both stromal cells and hematopoietic cells (e.g. DCs and macrophages) trans-present IL-15 to the Ly49<sup>-</sup> NK cells which then differentiate into Ly49<sup>+</sup> NK cells and become IL-15 dependent. This population leaves this "niche" (through an unknown mechanism but possibly mediated by IL-15) to allow further occupancy by NKp's and Ly49<sup>-</sup> NK cells. The Ly49<sup>+</sup> NK population then occupies a "hematopoietic niche" that includes DCs and macrophages where NK cells then differentiate into mature NK cells.

profile. Nevertheless, I have found IL-15 is critical for the proliferation of mature NK cells in the BM but not in the periphery. This IL-15-independent proliferation observed in the periphery may be due to the cellular niche, constraints on IL-15-mediated cellular expansion or other factors regulating proliferation. For instance, both NK and iNKT cells reside in high numbers in the liver but differences in BrdU incorporation was observed for both cell-types in IL-15R $\alpha$ <sup>-/-</sup> and WT mice. In hepatic NK cells, the incorporation of BrdU by NK cells was similar in both IL-15R $\alpha$ <sup>-/-</sup> and WT mice whereas hepatic iNKT cells in IL-15R $\alpha$ <sup>-/-</sup> mice had a significant decrease in BrdU incorporation in comparison to WT iNKT cells. Thus, the cellular niche may influence the function of IL-15 in the various IL-15-dependent populations.

Additionally, I revealed IL-15 regulates NK cell differentiation at two different levels of development. The first differentiation event regulated by IL-15 is the acquisition of the various Ly49 receptors. A past report had hinted at the notion of IL-15 regulating Ly49 expression but the interpretation of that study suggested IL-15R $\alpha$  expression by NK cells was crucial in this differentiation process<sup>67</sup>. Using mixed BM chimeras, I have completely ruled out the notion that IL-15R $\alpha$  expression by NK cells is required for the induction of the Ly49 repertoire. In human NK cells, IL-15 has also been shown to regulate KIR expression via the transcription factor, c-myc<sup>133</sup>. Other transcription factors (Gata-3 and Runx1) have also been shown to regulate Ly49 receptors in murine NK cells<sup>55,58</sup>. Thus, it is crucial to decipher the transcriptional network regulating both activating and inhibitory Ly49 receptor expression and what role, if any, IL-15 has in regulating these transcription factors.

The second differentiation event regulated by IL-15 is the transition of mature CD11b<sup>Low</sup> NK cells into CD11b<sup>High</sup> NK cells. This differentiation event is very dependent on IL-15 trans-presentation by hematopoietic cells. Recently, it was also shown IL-15R $\alpha$  expression by either DCs or macrophages contributes to the generation of CD11b<sup>High</sup> NK cells<sup>122</sup>. Interestingly, the effector function of CD11b<sup>High</sup> NK cells is greater than that of CD11b<sup>Low</sup> NK cells<sup>47</sup>. Thus, it could be speculated that NK cell functional maturation occurs during this cellular differentiation. Since IL-15 induces effector functions and the transcription factors, MEF and CEBP- $\gamma$ , regulate effector function, IL-15 may regulate MEF and CEBP- $\gamma$ <sup>61,62</sup>.

With regards to IL-15 regulating Ly49 expression, could NK cell “education” be co-dependent on IL-15? This role is plausible since IL-15 signaling assist in the up-regulation of Ly49 receptors and can activate NK cells<sup>73</sup>. What's more, transgenic expression of Bcl-2 in IL-15R $\beta$ -deficient mice recovered NK cell numbers but did not recover the cytolytic activity of rescued NK cells suggesting IL-15R $\beta$  signaling is important for functional maturation<sup>69</sup>. Moreover, IL-15R $\alpha$  and MHC class Ia are expressed by both hematopoietic and non-hematopoietic cells which would enable either cell-type the ability to trans-present IL-15 and interact with the numerous Ly49 receptors. Collectively, this hints at a possible role of IL-15 signaling in the education of NK cells. Thus, it could be hypothesized that during IL-15 trans-presentation by an IL-15R $\alpha$ <sup>+</sup> cell (hematopoietic or non-hematopoietic), NK cell ‘education’ or the preparation thereof may take place, a concept previously thought to be mediated by MHC class Ia and an unknown activating signal.

#### 4.1.3 Conclusion.

In summary, I have revealed IL-15 trans-presentation by DCs contributes to the in vivo development and maintenance of functionally mature NK cells. My data highlight an important concept that the developmental and homeostatic niche of NK cells is not only driven by IL-15 but by distinct IL-15R $\alpha$ <sup>+</sup> cell-types that may be tightly regulated by the tissue microenvironment. This understanding of IL-15 trans-presentation by specific IL-15R $\alpha$ <sup>+</sup> cell-types can assist in the quest to restore, maintain, or differentiate NK cells after adoptive transfer or BM transplantation. Thus, the identification of the cell-types and the regulation of IL-15 trans-presentation by these cell-types may offer an invaluable tool that will allow for the manipulation of NK cells in vivo.

### **4.2 Summary of Chapter 3.**

#### 4.2.1 The source of IL-15 during iNKT cell development and homeostasis.

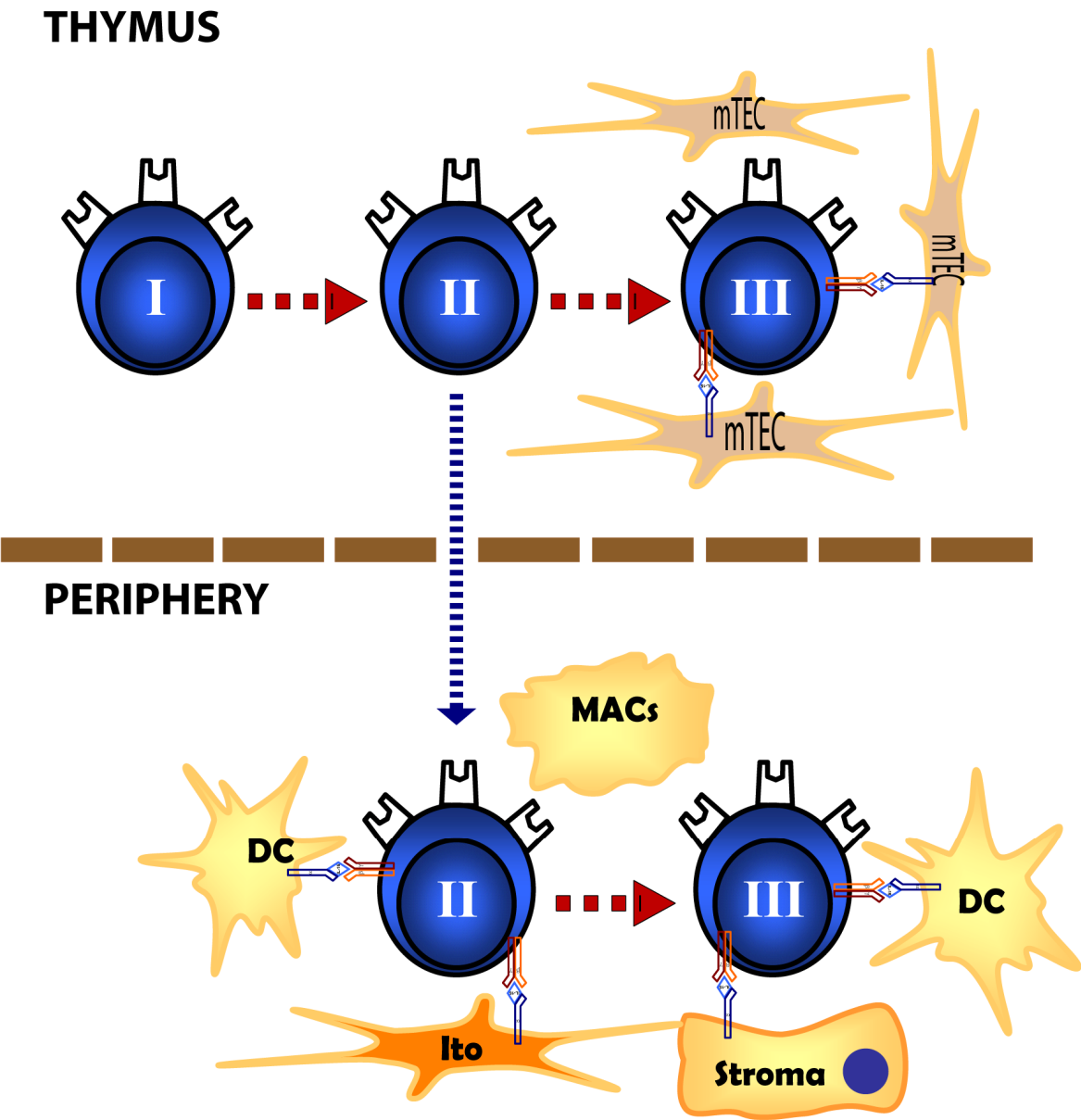
IL-15 has been shown to be critical for the development and homeostasis of iNKT cells; however, the cell-type providing IL-15 to iNKT cells was unknown.

Nevertheless, preliminary studies analyzing the cell-type trans-presenting IL-15 to NK1.1<sup>+</sup> T cells provided the first clues that both hematopoietic and non-hematopoietic compartments are crucial for iNKT cells<sup>29</sup>. Naturally, my experiments investigating IL-15 trans-presentation by hematopoietic and non-hematopoietic cells for invariant NKT cells revealed both compartments are equally important. Surprisingly, the tissue microenvironment dictated which cell-type was trans-presenting IL-15 to iNKT cells as depicted in **FIGURE 25**.

Briefly, the cell-type providing IL-15 to iNKT cells in the thymus was strictly of non-hematopoietic origin. A likely candidate is the IL-15-producing medullary thymic epithelial cells (mTEC). Although hematopoietic cells are present in the thymus they appear to have no role in maintaining thymic iNKT cells. This is likely due to various developmental events directing iNKT cells to a certain anatomical location in the thymus. In the thymus, the CD44<sup>High</sup> NK1.1<sup>+</sup> iNKT cells are dependent on IL-15<sup>34</sup>. This population of iNKT cells expresses CXCR3 which is actively involved in the retention of iNKT cells in the thymus<sup>116</sup>. mTECs have been shown to produce CXCR3 ligands which consequentially will retain iNKT cells in the thymic medullary epithelium. Therefore, the dynamics of the tissue microenvironment and the events occurring during iNKT cell development control the cell-type trans-presenting IL-15 to thymic iNKT cells.

Unlike thymic iNKT cells, peripheral iNKT cells can receive IL-15 trans-presented by either hematopoietic or non-hematopoietic cells. In this study, I have identified DCs as a major hematopoietic cell-type involved in hepatic iNKT cell homeostasis. The interaction of iNKT cells with DCs are likely due to both cell-types being in the same anatomical location. Specifically in the liver, both cells are found in the liver sinusoids so their interaction can be anticipated<sup>147</sup>. IL-15R $\alpha$  BM chimeras also revealed hepatic iNKT cell numbers were controlled by IL-15R $\alpha$ <sup>+</sup> non-hematopoietic cells, which are likely hepatic stellate cells (Ito cells). Similar to DCs, Ito cells can produce IL-15 and have been shown to drive the homeostatic proliferation of

FIGURE 25. Regulation of iNKT cell development.



**FIGURE 25. Regulation of iNKT cell development.**

An illustrative model is shown depicting the various hematopoietic and non-hematopoietic cell-types trans-presenting IL-15 to developing iNKT cells in the thymus and periphery. In the thymus, medullary thymic epithelial cells trans-present IL-15 to stage 3 (CD44<sup>High</sup>NK1.1<sup>+</sup>) iNKT cells that have migrated to the thymic medullary epithelium. In the liver, both hematopoietic (DCs) and non-hematopoietic (Ito cells) compartments can trans-present IL-15 to stage 2 (CD44<sup>High</sup>NK1.1<sup>-</sup>) and stage 3 (CD44<sup>High</sup>NK1.1<sup>+</sup>) iNKT cells.



hepatic iNKT cells<sup>121</sup>. Ito cells are also situated in the liver sinusoids thus, capable of interacting with iNKT cells<sup>121</sup>. The presence of DCs and Ito cells in close proximity to hepatic iNKT cells provide likely clues on why this tissue site is so rich for iNKT cells and dependent on both cellular compartments for IL-15. So although thymic and hepatic iNKT cells receive IL-15 by different cell-types (mTEC, DCs, and Ito cells) these cells express similar co-stimulatory molecules<sup>118,121</sup>. Thus, dissecting the interaction of iNKT cells with mTEC, DCs, and Ito cells may reveal why iNKT cells appear to be functionally a heterogeneous population of invariant T cells.

#### 4.2.2 The function of IL-15 during iNKT cell development and homeostasis.

IL-15 has previously been shown to be critical for the survival and expansion of iNKT cells<sup>34,111</sup>. On the other hand, the role of IL-15 in other aspects of iNKT cell biology such as function and activation has remained unexplored. During iNKT cell development, thymic and peripheral iNKT cells undergo cellular survival and multiple developmental events including cellular expansion, differentiation and retention (thymic iNKT cells). Of these events, I found IL-15 mediated the survival of thymic iNKT cells by maintaining high levels of the anti-apoptotic molecule, Bcl-2. In the periphery, IL-15 was vital for both survival (Bcl-2 mediated) and proliferation of iNKT cells. Once again, I found that the location was an instrumental aspect in determining the effects of IL-15 in iNKT cell biology.

Additionally, I revealed IL-15 is critical in both the functional maturation and activation of iNKT cells. T-bet is known to induce the terminal maturation of iNKT cells by regulating the expression of various cell surface receptors like NK1.1, CD122, and CXCR3 as well as effector molecules like IFN- $\gamma$ , granzyme b and perforin <sup>116</sup>. Interestingly, iNKT cells derived from IL-15R $\alpha^{-/-}$  mice expressed low levels of T-bet and CD122, and upon activation produced very low levels of IFN- $\gamma$  (hepatic iNKT cells). Surprisingly, normal expression levels of NK1.1 and CXCR3 (thymic iNKT cells) were observed on iNKT cells developing in IL-15R $\alpha^{-/-}$  mice. In the thymus, I speculate that once T-bet is expressed it initiates the transcription and subsequent expression of NK1.1, CXCR3, and CD122 by iNKT cells. CXCR3 expression directs iNKT cells to the thymic medullary epithelium where they receive an IL-15 signal. Seeing IL-15 increases T-bet levels which further enhances CD122 expression consequently making iNKT cells more responsive to IL-15.

As previously mentioned, T-bet also regulates IFN- $\gamma$ , granzyme b and perforin expression <sup>116</sup>. Whether IL-15 affects the effector function of thymic iNKT cells is unclear given that thymic iNKT cells do not respond to  $\alpha$ GalCer in vivo. It is plausible to think thymic iNKT cells are just not functionally mature. On the contrary, it is clear that IL-15 signaling is essential for the functional maturation of hepatic iNKT cells. But why would IL-15 turn on the effector program only in the periphery but not in the thymus? Perhaps, this is why iNKT cells have evolved to obtain IL-15 from different cellular sources depending on the tissue site they reside. So although non-hematopoietic cells can provide IL-15 to iNKT cells in the periphery it is

possible IL-15 trans-presentation by hematopoietic cells endows iNKT cells with effector function. So once again, the tissue microenvironment where iNKT cells reside determines the function of IL-15.

#### 4.2.3 Conclusion.

In summary, the tissue microenvironment influences iNKT cells at different stages of development as well as regulates their function. By restricting IL-15R $\alpha$  expression to certain cellular compartments, I was able to better define the cell-types trans-presenting IL-15 to iNKT cells in the various tissues. Depending on the tissue, IL-15 regulates the survival and/or proliferation of iNKT cells as well as impacts the functional status of iNKT cells. Overall, identification of the various cell-types providing IL-15 to iNKT cells could prove invaluable for improving iNKT cell adoptive immunotherapy and manipulating the immune response in specific tissue microenvironments.

### **4.3 General Discussion.**

#### 4.3.1 Exploitation of NK and iNKT cells.

Recognizing the factors that assist in the generation of a functional immune system can easily be utilized in translational research. This vital knowledge enables scientists and clinicians the ability to manipulate lymphocytes for adoptive transfer therapies treating immunocompromised patients or individuals suffering from life threatening diseases. Additionally, understanding the reconstitution of the immune system is extremely important in HSCT patients who are susceptible to numerous

opportunistic infections. Two immune cells that show great potential in the clinic are NK and iNKT cells as both have been shown to be crucial in tumor and viral immunity<sup>38,148</sup>.

Currently, graft-versus host disease (GVHD) is a major hindrance in cancer patients receiving allogeneic hematopoietic stem cell transplantation (HSCT)<sup>149</sup>. GVHD is a life-threatening condition that occurs when donor immune cells launch a massive immunological attack on the various tissues of the recipient. GVHD is initiated when the patient receives a conditioning regimen (chemotherapy or irradiation) to deplete the host immune cells. This treatment damages the host tissue which inevitably causes the activation of donor antigen presenting cells (e.g. DCs) via the release of proinflammatory cytokines being produced by damaged tissues and the release of lipopolysaccharide (LPS) from the intestine. These activated DCs present host antigen to donor T cells which then attack various tissues in the host. In addition, activated donor T cells can also cause Graft versus tumor (GVT) but at the cost of normal tissue damage. Depletion of donor T cells before transplantation has reduced the risk of GVHD; however, the reoccurrence of this condition is substantially higher<sup>150</sup>. Thus, immune effector cells, like T cells, are essential for GVT but preventing GVHD is crucial for patient health and survival.

NK cells have recently been shown to provide the GVT effect with little to no GVHD<sup>151</sup>. The basis behind this GVT effect was dependent on NK cells expressing killer-cell immunoglobulin-like receptors (KIR)<sup>152</sup>. KIRs can be activating or inhibitory and

the inhibitory KIRs interact with various human leukocyte antigens (HLA). This KIR-HLA interaction is analogous to the Ly49-MHC interaction in mice which results in NK cell education <sup>44,153</sup>. Thus, educated NK cells can detect “missing self” via KIR-HLA incompatibility which would result in target cytotoxicity. In humans and mouse models, the presence of NK cells have been shown to enhance BM engraftment as well as display strong anti-tumor activity in the recipient receiving both T cell-depleted allogeneic HSCT and NK cells infusion <sup>154</sup>. Suggestions have been made that NK cells assist in graft acceptance by killing donor T cells, inducing GVT by killing HLA-mismatched tumors, and preventing GVHD by killing host DCs and prevention of donor T cell activation <sup>151,152</sup>. Thus, understanding what regulates KIR expression in NK cells is essential for GVT and studies like mine provide key clues in enhancing our understanding in NK cell receptor regulation.

Regarding iNKT cells in the clinic, the ability to skew the cytokine profile of iNKT cells with various modified glycolipids and their capacity to cross-talk with DCs and macrophages, highlights their potential in various pathological states driven by distinct cytokines (Th1 versus Th2). Thus, the proper manipulation of iNKT cells can prove beneficial in the prevention of various autoimmune diseases and various cancers <sup>148</sup>. Additionally, both host and donor iNKT cells have also been shown to be critical in preventing GVHD and inducing GVT, respectively. Preventing GVHD is due to iNKT cells ability to produce Th2 cytokines <sup>155</sup> and the conditioning regimen enhances host iNKT cell function <sup>156</sup>. On the other hand, donor iNKT cells have been shown to induce GVT in the absence of GVHD <sup>157</sup>. Overall, the significant role

of NK and iNKT cells in immunosurveillance makes knowledge of their immunological niche very important in understanding their roles at steady-state and pathophysiological conditions, critical but lacking knowledge.

The foundation of generating functionally mature NK and iNKT cells relies on the various cellular and molecular cues acquired throughout development. Additionally, the occupation of various tissue microenvironments by NK and iNKT cells provides an additional factor contributing to these functional attributes. One such factor being provided by the tissue microenvironment is the cytokine IL-15. However, how IL-15 regulated these various events and what cell-type provides IL-15 for these two different IL-15-dependent populations was unknown. This lack of knowledge and the fact that the requirement of IL-15 is similar in NK and iNKT cell biology demanded immediate attention.

Therefore, my goal was to identify the cellular cues which govern the generation of these two lymphocytes thus utilizing these cues for immunological control or manipulation. Independently, each of my aims has contributed to the understanding and biological diversity of IL-15 in generating a functional immune system. Specifically, I have demonstrated that IL-15 is an essential factor involved in the differentiation, functional maturation, proliferation, survival and homeostasis of NK and iNKT cells. In conclusion, this study will prove pioneering and exploitable, as it utilized established models and techniques to address when and where IL-15 trans-presentation is important in NK and iNKT cell biology.

## CHAPTER 5

### MATERIALS AND METHODS

#### 5.1 Mice.

C57BL/6J (CD45.1 and CD45.2) mice were purchased from The Jackson Laboratories (Bar Harbor, ME) and NCI (Bethesda, MD). IL-15R $\alpha^{-/-}$  mice were generously provided by A. Ma (UCSF) and backcrossed to C57BL/6 mice 15 generations. CD11c/IL-15R $\alpha$  Tg mice backcrossed to the IL-15R $\alpha^{-/-}$  background were generated in our laboratory and described elsewhere<sup>35</sup>. Tg founder line no. 1 was used throughout this study and characterized as having IL-15R $\alpha$  expression restricted to CD11c<sup>+</sup> cells. Tg positive mice were identified by PCR of tail DNA and confirmed by IL-15R $\alpha$  staining on CD11c<sup>+</sup> cells. Mice were 6-14 wk of age when used in the various experiments. All mice were maintained under specific pathogen-free conditions at the University of Texas M.D. Anderson Cancer Center in accordance with the Institutional Animal Care and Use Committee guidelines.

#### 5.2 Generation of BM chimeras.

BM was collected from the tibia and femurs of IL-15R $\alpha^{-/-}$  (CD45.1 or CD45.1/CD45.2) and C57BL/6J (CD45.1) mice by crushing with mortar and pestle. T cells were removed from the isolated BM cells by treating with anti-Thy1 mAb (30 H12), followed by the incubation of Low-Tox-M rabbit complement (Cedarlane Laboratories) for 1 h at 37 °C. IL-15R $\alpha^{-/-}$  (CD45.2) and C57BL/6J (CD45.2)

recipients were irradiated with 1000 cGy and injected i.v. with  $3 \times 10^6$  BM cells. In some experiments, BM cells from IL-15R $\alpha^{-/-}$  (CD45.1/CD45.2) and C57BL/6J (CD45.2) were mixed in equal proportions before injecting into irradiated C57BL/6J (CD45.1) recipients. Chimera mice were analyzed for the presence of donor NK cells in the BM, liver, and spleen 8–12 wk later after complete BM reconstitution.

### 5.3 Lymphocyte isolation and Flow Cytometry.

BM cells were isolated by flushing a femur and tibia with HBSS/HGPG and filtering through a 70- $\mu$ m nitex. Thymus and spleens were homogenized in HBSS containing Hepes, L-glutamine, gentamycin and pen-strep (HGPG) using frosted slides and filtering through 70  $\mu$ m nitex. Livers were collected after intracardiac perfusion with PBS containing heparin, minced, and digested in RPMI with 10% FBS and 150 U/ml collagenase (Invitrogen). Remaining tissue was pressed through a 70 mm cell strainer and lymphocytes were isolated with a 44-67% Percoll (Amersham) gradient. When analyzing NK cells, single cell-suspensions were stained with the indicated Abs obtained from BD Biosciences (CD3 (17A2)-FITC, CD122-PE, NK1.1-PerCP-Cy5.5, CD11b-PE-Cy7, CD49b (DX5)-PE, Ly5.2 (CD45.1)-PE, CD94-PE, and CD69-Pe-Cy7) or eBioscience (Ly49 C/I/F/H-FITC, Ly49 G2 (4D11)-FITC, Ly49 I (YLI-90)-PE, Ly49 A/D-PE, CD49b (DX5) allophycocyanin, KLRG1 allophycocyanin, NKG2D (CD314) allophycocyanin, Ly49 H (3D10)-AlexaFluor 647, CD107a (1D4B)-AlexaFluor 647, and NKG2 A/C/E (20d5)-biotin) and Biolegend (Ly49 A PB, CD43 allophycocyanin and TCR $\beta$  allophycocyanin AlexFluor 750). With regards to iNKT cells, cells were first stained with CD1d-PBS57 loaded tetramer allophycocyanin-conjugated (NIH Tetramer Facility) for 30 minutes in the dark at 37 °C then washed



and followed by other indicated cell surface markers. The indicated antibodies were obtained from BD Bioscience (San Jose, CA) CD3 (17A2)-FITC, TCRb (H57)-FITC, CD45.2 (104)-FITC, CD45.1 (A20)-PE, CD44 (IM7)-PE, CD122 (TM-b1)-PE, IFN- $\gamma$ -PE, NK1.1 (PK136)-PE, NK1.1-PerCP-Cy5.5, CD45.2 (104)- PerCP-Cy5.5, CD4 (RM4-5)-PE-Cy7 or eBioscience (San Diego, CA) T-bet-PE (eBio4B10), CD45.2 (104)-Allophycocyanin-Cy7, CD44 (IM7)-eFluor™ 450; and Biolegend (San Diego, CA) CD94(18d3)-Pacific Blue, CD45.1 (A20)-Pacific Blue and CD45.1 (A20)-Allophycocyanin-Cy7 and TCR $\beta$  Allophycocyanin-AlexFluor 750. To detect Bcl-2 in iNKT cells, intracellular staining was performed using BD cytofix/cytoperm kit (BD Bioscience, San Jose, CA) and stained with anti-mouse Bcl-2 FITC. To analyze T-bet expression by iNKT cells, intracellular staining was performed using eBioscience Foxp3 staining buffer set. All samples were collected on a LSR II Flow Cytometry (BD Bioscience) and data were analyzed with FlowJo software (Treestar, San Carlos, CA).

#### 5.4 Cell cycle labeling.

For long-term labeling in NK cells, mice were administered water supplemented with BrdU (0.8 mg/ml). BrdU-supplemented water was changed every 2 days for 3 wk. Long-term BrdU labeling in iNKT cells, mice were administered water supplemented with BrdU (0.8mg/ml). BrdU supplemented water was changed every day for one week. BrdU Flow kit (BD Pharmingen) and protocol was used to analyze NK and iNKT cell incorporation of BrdU. Cell surface stains included markers to identify individual development stages for NK cells and iNKT cells. Cells were then fixed

and permeabilized using BD cytofix/cytoperm buffer, followed by DNase (30 µg) treatment. Cells were then stained with anti-BrdU FITC for 20 min at room temperature and analyzed as stated above. Cells were then stained with anti-BrdU FITC for 20 min at room temperature and analyzed as stated above.

### 5.5 Adoptive transfer.

To enrich for NK cells, 10–15 spleens were pooled from C57BL/6J (CD45.1) mice and incubated with rat anti-CD8 (2.43), anti-CD4 (Gk1.5), anti-CD19 (1D3), and anti-MHC II (M5), followed by washing and a subsequent incubation with goat anti-rat Dynabeads (DynaL Biotech). Magnetic-coated cells were then removed with a magnet. Enriched cells were CFSE-labeled, and  $7\text{--}10 \times 10^6$  cells were injected i.v. into IL-15R $\alpha^{-/-}$  (CD45.2), CD11c/IL-15R $\alpha$  Tg (CD45.2), and C57BL/6J (CD45.2) mice. Three weeks later, mice were sacrificed, the BM, liver, and spleen were harvested, and donor cells were analyzed for cell surface phenotype and cellular division by flow cytometry. For iNKT cell enrichment, 10 -15 thymuses were pooled from C57BL/6J (CD45.1) mice and incubated with rat anti-CD8 (2.43), anti-CD19 (1D3), anti-MHC II (M5) followed by washing and a subsequent incubation with goat anti-rat Dynabeads and then magnetic cells were removed as previously described. In experiments examining homeostasis, enriched cells were labeled with the fluorescent cell staining dye, Carboxyfluorescein succinimidyl ester (CFSE), and  $5\text{--}8 \times 10^6$  cells were injected i.v. into IL-15R $\alpha^{-/-}$  (CD45.2), CD11c/IL-15R $\alpha$  Tg (CD45.2) or C57BL/6J (CD45.2) mice. Seven days after transfer, tissues were harvested and

analyzed for the presence of donor cells and CFSE dilution by flow cytometry. For  $\alpha$ GalCer stimulation, mice were sacrificed 2 days after transfer of donor cells.

#### 5.6 DC-NK cell coculture system.

Spleens from four to five CD11c/IL-15R $\alpha$  Tg or WT mice were pooled and enriched for NK cells as previously described above. Enriched cells were stained with CD3, NK1.1, Ly49 C/I/F/H, and Ly49 A/D and then sorted on the FACS Aria II cell sorter (BD Biosciences). Sorted population contained a NK1.1<sup>+</sup> Ly49<sup>-</sup> CD3<sup>-</sup> cells, which were used for coculturing with C57BL/6J and IL-15R $\alpha$ <sup>-/-</sup> DC. DC derived from C57BL/6J and IL-15R $\alpha$ <sup>-/-</sup> mice were generated by culturing BM-derived cells with GM-CSF at 1000 U/ml supplemented complete media (RPMI 1640, 10% heat-inactivated FCS, 10 mM HEPES, and 50  $\mu$ M 2-ME). Cells were washed and fed every 2 days for 7 days. BM-derived DC were then collected and cocultured at a 1:3 ratio with NK cells negative for Ly49 C/I/F/H and A/D. After 5 days, NK cells were then stained for CD3, NK1.1, Ly49 C/I/F/H, and Ly49 A/D cell surface markers and analyzed by flow cytometry as described previously.

#### 5.7 IFN- $\gamma$ and CD107 $\alpha$ stimulation assay.

Spleens from four to five CD11c/IL-15R $\alpha$  or WT were pooled, enriched for NK cells as previously described above. NK1.1<sup>+</sup>CD3<sup>-</sup> cells were sorted from the enriched fraction on the FACS Aria II cell sorter (BD Biosciences) and incubated for 5 h in the absence or presence of plate-bound anti-NK1.1 Ab at 37°C as described previously

<sup>44</sup>. Cultures were supplemented with 1  $\mu$ l of CD107a (1D4B) Ab and 1  $\mu$ l of GolgiPlug (BD Biosciences). The cells were then fixed and permeabilized using BD Cytotfix/Cytoperm buffers and stained with PE-conjugated anti-IFN- $\gamma$  or control Ig mAb (BD Biosciences) as well as CD3 and NK1.1 and analyzed via flow cytometry.

#### 5.8 In vivo $\alpha$ -Galactosylceramide and IL-15/IL-15R $\alpha$ -Fc Complex stimulation.

$\alpha$ -Galactosylceramide (2  $\mu$ g/mouse) (Funakoshi co., Ltd Tokyo, Japan) diluted in 1x PBS was injected i.v. into the various mice. Tissues were collected and processed as previously described 2.5 hrs post-injection. Single cell suspensions were supplemented with 2  $\mu$ L of GolgiStop (BD Bioscience, San Jose, CA) for 1 h at 37  $^{\circ}$ C. After staining for cell surface markers, cells were then fixed and permeabilized and stained with PE-conjugated anti-IFN- $\gamma$  as previously described and analyzed via flow cytometry. When analyzing the effects of IL-15 signaling in IL-15R $\alpha^{-/-}$  mice prior to  $\alpha$ GalCer stimulation (sacrificed 2d later) or T-bet expression (sacrificed 1d later), each mouse received an i.p. injection of 2.5  $\mu$ g of recombinant murine IL-15 (PeproTech, Rocky Hill, NJ) precomplexed to 15 $\mu$ g of recombinant IL-15R $\alpha$ /Fc chimera (R&D Systems) <sup>137</sup>. To briefly explain the formation of the complex, 2.5  $\mu$ g of recombinant murine IL-15 resuspended in 1x sterile PBS was mixed and incubated for 30 minutes at room temperature with 15  $\mu$ g of recombinant IL-15R $\alpha$ /Fc chimera resuspended in 1x sterile PBS supplemented with 0.1% BSA.

### 5.9 Statistical Analysis.

Graphs generated display the mean  $\pm$  standard error of the mean (S.E.M.) and were obtained using GraphPad Prism software (GraphPad Software, La Jolla, CA).

Statistical significance was assessed by performing an unpaired Student's t test.

Data collected from experimental mice (chimeras where IL-15R $\alpha$  was restricted to either hematopoietic or non-hematopoietic cells, or Tg mice) was compared to the data generated from mice completely lacking IL-15R $\alpha^{-/-}$  unless the data was only comparing IL-15R $\alpha^{-/-}$  and WT control mice.

## **CHAPTER 6**

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## **CHAPTER 7**

### **VITA**

Eliseo Fernando Castillo was born in Hobbs New Mexico on August 13<sup>th</sup> 1980 to Isidro and Juanita Castillo. In 1998, he completed the necessary requirements to obtain his high school diploma from Hobbs High in Hobbs New Mexico. After graduation, he went on to pursue a Bachelor of Science in Biology from Eastern New Mexico University in Portales New Mexico, which he completed in December 2003. The following January he entered into the Chemistry graduate program at Eastern New Mexico University and obtained a Master of Science in Chemistry under the guidance of Dr. Newton Hilliard in July 2005. In August of 2005, he entered into the Ph.D. degree program at The University of Texas Health Science Center at Houston Graduate School of Biomedical Sciences. In June of 2006, he began his Ph.D. training under the supervision of Dr. Kimberly S. Schluns in the Department of Immunology at the University of Texas M.D. Anderson Cancer Center.

## CHAPTER 8

### PUBLICATIONS

1. **E.F. Castillo**, L.F. Acero, S.W. Stonier, D. Zhou and K.S. Schluns (2010) Thymic and peripheral microenvironments differentially mediate development and maturation of iNKT cells by IL-15 transpresentation. *Blood*, DOI: 10.1182/blood-2010-03-277103.

2. **E.F. Castillo**, S.W. Stonier, L. Frasca and K.S. Schluns (2009) Dendritic cells support the in vivo development and maintenance of NK cells via IL-15 trans-presentation. *J. Immunol.* 183(8): 4948-4956.

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